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Effect of atmospheric humidity during ripenning on the pod setting and seed germination in excised alsike clover (*Trifolium hybridum* L.)

Kaoru Ehara

Introduction

Certain regions of the world because of favorable climatic condition are better adapted to growing the legume seed than other sections are. One of the favorable climatic factors is dry weather. It is known that the soil and atmospheric humidity affect the seed setting of legumes and a few investigations have been done with legumes on this subject. It would appear that atmospheric humidity affects the hard seed percentage of legumes, however there are few reliable studies on this subject. This fact probably due to the difficulty of atmospheric humidity control within the cabinet for growing plants.

Recently, phytotrons are building in many countries including Japan, but few studies have been done with legumes on the effect of atmospheric humidity on the seed production and germination. In Japan, atmospheric humidity in season of cross-pollination and ripenning of legumes is high and the author is interesting in this subject.

Review of literature

There are a few reports on the effect of atmospheric humidity on the seed production of legumes. Alter (1920) stated that observations have indicated that the production of alfalfa seed is best in area which the air humidity is usually low and the soil moisture below optimum. Blinn (1920) concluded that dry climatic conditions with high temperatures seem to be among the most essential requirements for seed

production of alfalfa. Hollowell (1929) found in his experiment that atmospheric humidity does not affect the setting of red clover seed under greenhouse or field conditions.

Grandfield and Zink (1937) have used the chamber for the control of air temperature and relative humidity, in which sulfuric acid was used to control humidity. This equipment permitted the studies to be carried on in natural light under greenhouse conditions. According to Grandfield (1945) the number of flowers setting pods of alfalfa increased as the relative humidity decreased from 90 to 10 per cent. At 80°F., the difference between the percentage of pod set at 10 per cent relative humidity and the percentage at 90 per cent was 24.

There are few studies on the effect of atmospheric humidity during ripenning of seed on the germination percentage of legumes. According to Jones (1928), Hiltner (1903) thinks that crops ripenning in a dry climate or in dry seasons show a greater proportion of hard seed. Jones stated that the hard seed percentage in *Vicia villosa* can be distinctly influenced by the storage temperatures and humidities, high humidity usually decreasing the proportion of hard seed through its influence on the plant. The data reported in his paper shown considerable variation in the hard seed percentages in *Vicia villosa* from different sources. Wheeler (1950) stated that in certain forage seeds, hard seed percentage varies with (1) species or variety (2) stage of maturity, and (3) conditions under which produced.

Materials and methods

Stems bearing florets of alsike clover were gathered from plants growing in pots. They were then brought to the laboratory, any withered lower leaves were removed. The stems ranged from 15 to 16 cm. in length and each stem was bearing a head and two leaves.

In the first test, all wilted or unopened florets were removed and florets varied from 60 to 70 in number per head, and study was made in three replications using freshly open-pollinated florets. In the second test, wilted florets were used and in the third test, freshly opened florets were cross-pollinated by honey bees within bell jar without replication in each test. Analysis of variance was made in the first test.

The stems were placed in a glass container holding about 50 cc. of 1 per cent glucose solution. According to Battle (1949), 2 per cent sucrose solution was selected as the culture medium for red clover and about three-fourths of an inch of the cut end was charred in a Bunson flame. However, the author's preliminary trials indicated that 1 per cent glucose solution served as a satisfactory medium for keeping the

leaves of alsike clover alive and charring of the cut end was not necessary. Glucose solution was renewed every 3 days.

In the sampling of materials, the heterogeneity of alsike clover was under consideration, and the materials were gathered from same several plants in each test.

The glass containers were covered with bell jars, and dry or wet atmospheres which passed through the scrubbing bottles holding sulfuric acid or water were continually induced in bell jars at the rate of 700 to 1,400 cc. a minute. The apparatus was shown in Fig. 1.

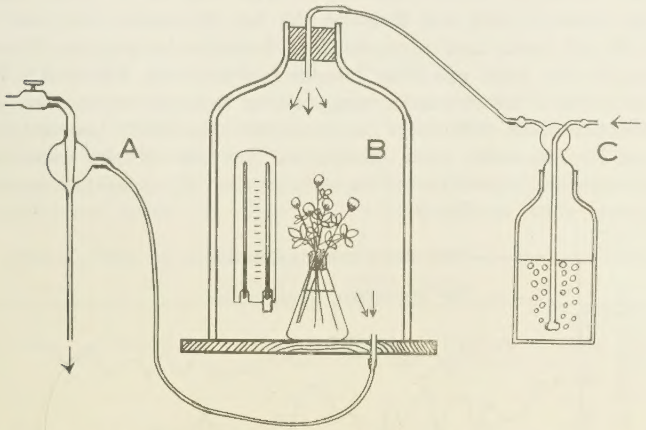

Fig. 1. Apparatus for control of atmospheric humidity.
A, Sucker; B, Bell jar; C, Scrubbing bottle.

Experiments were conducted in fields. To prevent the occurance of high temperatures within bell jar, it was necessary to place a shade over the apparatus and to dash water over bell jar when the sun was shining. In cold weather, it was necessary to pass air through ice blocks in order to obtain dry atmosphere.

The atmospheric humidity and the temperature within bell jar and field were measured every day. The season of experiments was from July 19 to August 8, 1956. The artificial control of humidity within bell jar was lasted for 2 weeks and from then glass containers were placed on a table in a moderately light laboratory.

When the heads were ripe, the seeds were removed and counted. The seeds were storaged in the decicators and germination tests with seeds obtained in the first test were made with three replications in 3, 8 and 11 months after harvesting. Apparent germination percentages are those calculated on the basis of the number of seeds planted. Apparent germination percentages are used in the results and discussion.

Results

1. *Atmospheric temperature and humidity*

One of the measurement results of atmospheric temperature and humidity within bell jars at 9 a.m., 2 p.m., and 6 p.m. was shown in Fig. 2. Atmospheric temperature was measured by mercury thermometer. Temperatures in field conditions during the experiment were not constant and therefore, as shown in Fig. 2, atmospheric temperature and humidity within apparatus were not constant. However, relative atmospheric humidity within apparatus varied from 85 to 95 per cent in the high humidity plot and those of the low humidity plot was from 50 to 80 per cent, and remarkable differences in relative humidity between in the high and low humidity plots were obtained. There were no marked differences in temperatures between in the two plots. It seemed that these differences in atmospheric humidity between in the high and low humidity plots served the purpose of the experiment. Temperature and humidity within other series of apparatus were approximately same as Fig. 2.

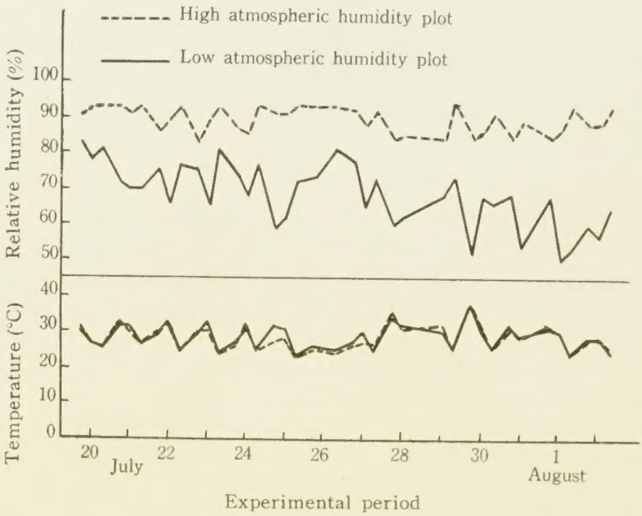

Fig. 2. Atmospheric temperature and humidity within bell jars (A–b).

2. *Observation during experiment*

The leaf color of alsike clover in the low humidity plot became paler green than that of the high humidity plot at 2 to 3 days after beginning of treatment, but at last of the experiment the latter lost the greeness and the margin parts of leaf were pale green. The florets

in the low humidity plot were of a pale brown color, while those of the high humidity plot were dark brown in color.

The transpiration was more remarkable in the low humidity plot than in the high one. At the beginning of experiment, the decreases in glucose solution were 35 to 40 cc. per glass container holding 5 stems every 3 days in the low humidity plot, whereas the corresponding numbers were 15 to 20 cc. for the high humidity plot. Transpiration of alsike clover decreased with the lapse of time.

3. Per cent pod set

The pod setting data for the first test are given in Table 1. Pod set of freshly open-pollinated florets on excised alsike clover varied from 34.1 to 61.3 per cent and general speaking, these percentages were somewhat lower than that of open-pollinated florets in the field conditions, whose value was 61.1 per cent. However, it seemed that the experiments were carried out successfully. As shown in Table 1, atmospheric humidity did not significantly affect the pod setting of freshly open-pollinated florets on excised alsike clover stem.

Table 1. Effect of atmospheric humidity on the pod setting of freshly open-pollinated alsike clover.

Apparatus	Treatment	Number of plants	Number of heads	Number of florets	Number of florets setting pods	Per cent pod set
A-a	Low humidity	4	15	543	329	60.5
	High humidity	4	20	1,104	677	61.3
A-b	Low humidity	4	30	1,924	799	41.3
	High humidity	4	26	1,518	605	39.9
A-c	Low humidity	7	46	2,675	911	34.1
	High humidity	7	54	2,822	1,207	42.8
Natural condition		12	117	7,888	4,816	61.1

Table 2 presents data on the pod set of the second test with the wilted florets, and the pod set on excised stems growing in the low

Table 2. Effect of atmospheric humidity on the pod setting of wilted florets of alsike clover.

Treatment	Number of plants	Number of heads	Number of florets	Number of florets setting pods	Per cent pod set
Low humidity	6	17	1,144	599	52.4
High humidity	6	18	1,189	633	53.2

humid atmosphere was 52.4 per cent and that of the high one was 53.2 per cent. Atmospheric humidity did not affect the pod setting in the second test.

Table 3 presents data on the pod set of the third test with florets which were cross-pollinated by honey bees in bell jar. The pod set of

Table 3. Effect of atmospheric humidity on the pod setting of alsike clover, cross-pollinated by honey bees in bell jar.

Treatment	Number of plants	Number of heads	Number of florets	Number of florets setting pods	Per cent pod set
Low humidity	3	25	958	210	21.9
High humidity	3	28	1,005	37	3.7

stems growing in the low humid atmosphere was 21.9 per cent, while the corresponding number for the high one was 3.7 per cent. The high atmospheric humidity significantly decreased the percentage of pod setting in the third test, and the percentages were lower than those of the first and second tests.

4. Germination of seeds

Table 4 presents data on the germination of alsike clover seed produced in the first test. In general, the germination percentages of alsike clover seeds obtained in the first test were very low when seeds

Table 4. Effect of atmospheric humidity on the germination of alsike clover seed produced in the first test (in per cent).

Apparatus	Replication of germination test	Season of germination test					
		First Oct. 26 to Nov. 5, 1956		Second Feb. 19 to Mar. 1, 1957		Third June 25 to July 5, 1957	
		Low humidity	High humidity	Low humidity	High humidity	Low humidity	High humidity
A-a	1	0	10	11	21	16	14
	2	1	8	7	18	18	12
	3	7	9	11	15	8	15
A-b	1	3	14	10	22	12	29
	2	0	18	13	23	15	29
	3	4	18	10	29	19	27
A-c	1	6	15	17	38	29	36
	2	0	30	30	41	21	41
	3	5	24	15	35	18	43
Average		2.9	16.2	13.8	28.0	17.3	27.3
Seed produced in natural condition		13.7		24.5		25.3	

were not scarified. However, the germination percentages of seeds produced in natural coditions were also low and those were 13.7, 24.5 and 25.3 per cent for the first, second and third germination tests respectively. The results of analysis of variance as applied to those data are presented in Table 5. As shown in Table 5, atmospheric humidity significantly affected the germination percentage of seeds produced in freshly open-pollinated florets on excised alsike clover stem and the germination percentages of seeds obtained in the high humid atmosphere were higher than those of the low one. The germination percentages of alsike clover seed significantly increased as the days after harvesting increased in the two cases.

Table 5. Analysis of variance of germination percentage.

Source of variation	Sum of squares	Degrees of freedom	Mean squares	Ratio of variance
Humidity (H)	1,873	1	1,873.00	121.07**
Season of germination test (S)	1,803	2	901.50	58.27**
Apparatus (A)	1,817	2	908.50	58.73**
Interaction H × S	56	2	28.00	1.81
H × A	354	2	177.00	11.44**
S × A	241	1	241.00	15.58**
H × S × A	28	7	4.00	
Error (E)	557	36	15.47	
Total	6,729	53		

** Significant at the 1-per cent level.

Discussion

In this experiment, although the humidity was not constant, the control of atmospheric humidity within the plant growth chamber was successful. If this apparatus was placed in the temperature controled large chamber, the experimental condition would be more satisfactory.

It is interesting to note that the transpiration was more remarkable in the low humidity plot than in the high one. The decrease in glucose solution in glass container was more marked in the low humidity plot than in the high humidity plot, whereas the decreases in solution in the glass containers without excised alsike clover stems were very small in the two plots. Therefore, the following observations seem justified under conditions of the experiments that the difference in solution decreases in glass containers between the high and low humidity

plots probably was due to the difference in the transpiration between the two plots.

According to the results of this experiment, atmospheric humidity did not significantly affect the pod set percentage of freshly open-pollinated or wilted florets on excised alsike clover stems. The results in this study agree with those of Hollowell's study (7) on red clover. However, when the stems with unopened florets were excised and covered with bell jar and were cross-pollinated by honey bees in bell jar, the activity of the insect was more vivid in the low humid atmosphere than in the high humid atmosphere. Most of the honey bees in the high humid atmosphere died soon after beginning of experiment. In the case of cross-pollination by honey bees in bell jar, pod set was markedly lower in the high humid atmosphere than in the low humid atmosphere. It seems reasonable to conclude that the inactivity of honey bees accounts for the lower percentage of pod setting in the high humid atmosphere. There is a good reason that certain regions of the world because of dry climate are better adapted to growing the seed than other sections are, and in dry climate pollinators such as honey and bumble bees play their role vividly.

According to Battle's report (2) on red clover, the seeds produced on excised stems were uniformly impermeable, and there were many impermeable seeds in alsike clover seeds produced in this experiment, nevertheless the alsike clover seeds were not always impermeable and some of them were permeable.

Although the germination percentage of seeds obtained in this experiment was low, but not especially lower in percentege of germination than that of seeds produced in natural conditions.

In general, alsike clover seeds produced in Kyūsyū, warm area in Japan, are low in percentage of germination, and it appears that high temperature partly affect germination of this legume seed.

Seeds from the high humid atmosphere were higher in percentage of germination than those from the low humid atmosphere, and this result agrees with Hiltner's observation that crops ripenning in a dry climate or in dry seasons show a greater proportion of hard seed.

In this experiment, the effect of afterripenning on the germination of alsike clover seed was remarkable. It seems that it will be necessary to scarify the alsike clover seeds produced in Kyūshū.

Summary

1. This paper gives the results of study on effect of atmospheric humidity during ripenning on pod setting on excised alsike clover stems and germination of seed produced in this experiment.

2. Excised stems were placed in a glass container holding about 50 cc. 1 per cent glucose solution and the glass containers were covered with bell jar, and dry or wet atmospheres which passed through scrubbing bottles holding sulfuric acid or water were continually induced into bell jars.
3. The transpiration was more remarkable in the low humidity plot than in the high humidity plot.
4. Atmospheric humidity did not significantly affect the pod setting of freshly open-pollinated or wilted florets on excised alsike clover stems.
5. Excised stems bearing unopened florets were covered with bell jar, and when the florets opened they were cross-pollinated by honey bees in bell jar. In this test, the percentage of pod setting was lower than in the case using freshly open-pollinated florets. The percentage was significantly lower in the high humidity plot than in the low one.
6. In general, the germination percentage of alsike clover seeds obtained in this experiment were very low when seeds were not scarified. However, the seeds were not always impermeable. Atmospheric humidity significantly affected the germination percentage of seed produced in freshly open-pollinated florets on excised stems, and the germination percentage of seeds obtained in the high humidity plot was higher than that of the low one.
7. The germination percentage of alsike clover seed significantly increased as the days after harvesting increased.

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Studies on the growth behaviour and quality of the forage crops in warm area in Japan

IV. Effect of temperature on the growth behaviour and chemical composition of corn, oats and red clover

Kaoru Ehara

Introduction

In Japan and foreign countries, there is a common belief that the forage crops grown under warm climate are inferior in quality than those grown under cold climate. However, there has not been any reliable research made on this subject in Japan, and a few conducted in foreign countries.

The author tried to investigate whether or not the above belief can be substantiated, and if so, to find the cause for, and counter-measure against it.

The geographical region to which each forage crop is adapted is limited by various climatic factors of which temperature is of major importance.

The effects of temperature on growth and chemical composition constitute one of the major problems in the management and utilization of forage crops. Therefore, an advanced knowledge of the temperature relations to forage crops should contribute to the solution of some of the problems of quality improvement of forage crops in warm area.

The species of forage crops included in investigations herein reported are corn (*Zea mays*), oats (*Avena sativa*), and red clover (*Trifolium pratense*).

Literature review

Many investigations have been done with forage crops on the effect of temperature on the growth, while there are a few studies on the

effect of temperature on the chemical, physical or anatomical properties of forage crops.

Walster (1920) who grew barley at two temperatures, found the lower temperature to induce a more upright character of growth because of a greater proportion of culm to leaf, a greater proportion of skeletal material in the leaf to all other plant substances, and a greater degree of lignification of conductive tissue than was found in plants grown under higher temperatures.

Newton (1922) found that he could distinguish cold-hardy from non-cold hardy varieties of wheat on the basis of their bound water content. He also observed the higher sugar content of the hardy variety as compared with that of the non-hardy, but assigned no particular significance to it. On the other hand, Meyer (1928) stressed the idea that soluble carbohydrates may increase frost resistance of plants by decreasing the precipitation of protein.

Hurd-Karrer and Dickson (1934), Lundegardh (1954), Nightingale (1934, 1935) and Tottingham (1923) all reported that plants accumulate less carbohydrates as the temperature becomes higher.

Delwiche and Tottingham (1930) measured the amount of crude protein in clover hay, corn, and barley grain in northern and southern Wisconsin. There were differences in latitude, altitude, length of season, number of fine days, amount of rainfall, and atmospheric moisture in those two places. According to them, the accepted opinion that northern crops are richer in protein than southern crops and therefore have higher feeding value was hardly substantiated. On the contrary, clover from northern Wisconsin had less protein than that from southern Wisconsin. Vinall and Hein (1937) showed that the southern limit of the region to which Kentucky and Canada bluegrass and orchard grass are adapted corresponds rather closely with the 60°F. isotherm. The northern limit for orchard grass corresponds roughly with the 45 F. isotherm, but the two bluegrasses are successfully grown much farther north.

According to Brown (1937), in Kentucky and Canada bluegrass and orchard grass, the percentage content of nitrogen-free extract declined with rising temperature but in Bermuda grass, this constituent increased in concentration as the temperature rose. In all four grasses, the crude fiber content increased in percentage as the temperature rose from 40° to 60°F. and changed little with further rises in temperature. The percentage of crude protein declined slightly in Kentucky and Canada bluegrass and orchard grass as the temperature increased from 40° to 60°F. and then increased slightly as the temperature rose above the optimum for growth. In Bermuda grass the crude protein content was much greater at 50° than 60°F.

Sullivan and Sprague (1949) found that protein metabolism was

characterized by an increase in the proportion of soluble nitrogen immediately after the leaves were clipped, and at the highest temperature this proportion continued to increase for a long period in the tops and stubble of perennial ryegrass. Other constituents, lignin, hemicellulose, and ash were not particularly affected by leaf removal or temperature except that their percentage were increased where soluble carbohydrates had been withdrawn. The losses were most rapid and extensive at the higher temperatures. The roots and stubble underwent rapid losses in sucrose and fructosan during the early part of experiment but these losses were partly replaced later under the low temperature conditions. Under the highest temperature these losses were not replaced but continued, especially in the roots, almost to the point of exhaustion and in some cases to the death of the roots. Carrol (1943) also stated that the detrimental effects of high temperatures are the result of a depletion of carbohydrates within the plant.

A review by Weinmann (1948) covers the general subject of reserves in grasses.

Ulrich (1955) found that the sugar beet were grown with an ample supply of water and nutrients in a controlled temperature greenhouse (days 23°C., nights 17°C.), and ripenning or "sugaring up" was induced by low night temperatures and nitrogen deficiency, i. e., changes in the external environment rather than by some internal self regulatory mechanism of the plant.

Experimental methods

In this experiment, thermo-regulated growth chamber was not used. The equipment used for the control of temperature consists of two glasshouses, the windows of the one were opened and those of the other one were closed. The air temperature in the former was lower than the latter, and the former is called the low temperature plot and the latter is called the high temperature plot in this experiment.

The red clover and oats cultures were grown in earthen flower plots, 30 cm. in diameter at the top, 25 cm. in diameter at the bottom, and 30 cm. deep. The corn cultures were grown in glaged earthenware pots, 25 cm. in diameter at the top and the bottom, and 31 cm. deep. The varieties used for this experiment were Northwestern dent corn, Carter's black tartar oats and Dollard red clover.

Corn, oats and red clover were planted directly in the potted soil, and the stands reduced to 2, 5 and 3 plants per pot as soon as the seedling appeared to be securely established in corn, oats and red clover respectively. Red clover was sown on December 20, 1955, corn and oats were sown on April 15, and 18, 1956 respectively. Ten cultures of

each species were established, and 5 cultures of each species were exposured to each different temperature plot from April 24 to June 30, 1956.

Corn plants were cut at silk stage, oats plants were cut at head stage and red clover plants were cut at bloom stage at the soil surface. Fresh and air dry weights are reported as g./pot. During growth period, the measurements of growth were made by each species. Stage of growth of corn and oats plants was indicated by the order of leaf instead of the number of days after sowing. Symbols of the emerging stage of leaf on main stem were the same as those in the previous report (Ehara et al. 1956).

With cut samples general chemical analyses were made and sugar and lignin content also was determined. In analysing lignin, colloid titration method as reported by Senju et al. (1955) was employed. The cut samples of corn were separated into leaves, stalks, ears and tassels, and leaves were separated into each leaf by the order of leaf. Oats were separated into leaves of main culm, main culms, heads of main culm and tillers, red clover samples were separated into leaves, stems and flower head. Analytical determinations were made on each organ from the three plants.

Results

1. Results of measuring temperature in the two glasshouses

The maximum and minimum temperatures and the temperatures at 9 a. m., and at 6 p. m. were measured in the glasshouses. As shown in Figures 1 and 2, in general the temperatures rose with advancing season, and this trend was most remarkable in the minimum temperatures. The maximum temperatures in the high temperature plot were about 1.3° to 5.0°C. higher than those in the low temperature one. The minimum temperatures in the high temperature plot were 1.0° to 2.5°C. higher than those in the low temperature plot up to last of June. The maximum and minimum temperatures in the two plots were higher than those in the field conditions, and those differences in temperatures between the glasshouses and field conditions were more remarkable in the maximum temperatures than in the minimum temperatures.

The curves in Figure 2 show that air temperatures at 9 a. m. are 1.8° to 4.0°C. higher in the high temperature plot than in the low temperature one throughout experimental period. The air temperatures at 6 p. m. are 0.6° to 4.0°C. higher in the former than in the latter.

As above mentioned, a relatively warm condition and a relatively cool condition were obtained by these temperature treatments, but the true temperatures in the two plots were extraordinarily higher than those in the field conditions.

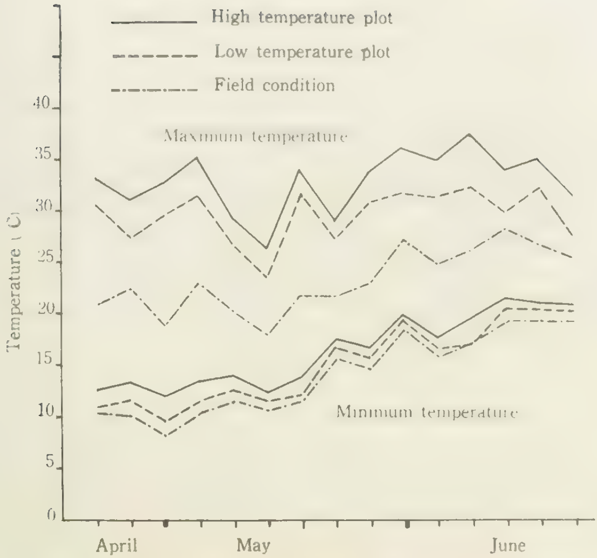


Fig. 1. Maximum and minimum temperatures in the two glasshouses.

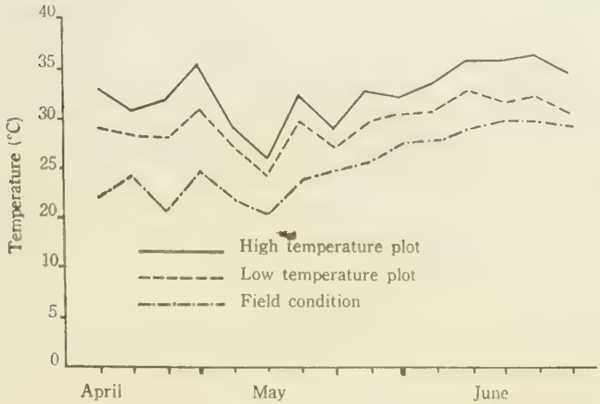


Fig. 2. Temperatures at 9 a.m. in the two glasshouses.

The soil temperatures at depth of 5 cm. in the pot were measured at 9 a.m., 2 p.m. and 6 p.m. and Table 1 shows the results.

As shown in Table 1, soil temperatures in the high temperature plot were a little higher than those in the low temperature plot.

The relative atmospheric humidities in the two plots were measured,

and there were not any remarkable differences between the high and low temperature plots.

Table 1. Soil temperatures in the pot at a depth of 5 cm.

Period	High temperature plot			Low temperature plot		
	9 a.m.	2 p.m.	6 p.m.	9 a.m.	2 p.m.	6 p.m.
	°C	°C	°C	°C	°C	°C
June 11-15	24.2	31.4	29.1	22.2	28.7	26.4
June 16-20	25.0	29.1	28.6	23.6	27.4	27.3
June 21-25	25.1	28.6	28.0	23.6	26.9	26.2
June 26-30	23.5	—	26.9	22.6	—	25.8

2. Growth behaviour and yields

a. Corn.

Leaf emerging stage.—The plant grown in the high temperature plot was a little faster in the appearance of leaves on the main stalk than the plant grown in the low temperature one, and as shown in Figure 3, the former was 3 days faster in the appearance of 15th leaf

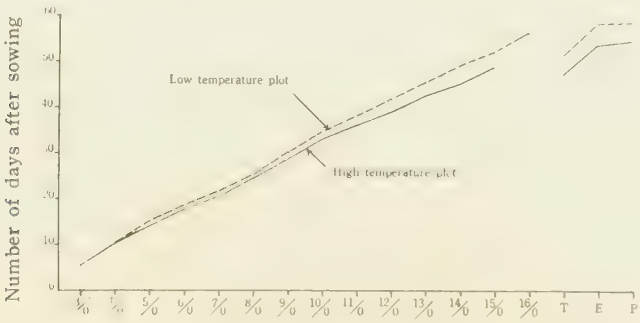


Fig. 3. Effect of temperature on the appearance of leaves on the main stalk of corn.

T = Beginning to tassel. E = End of tasseling.
P = Beginning to shed pollen.

(15/0) than the latter. The beginning to tassel and to shed pollen was 3 to 5 days faster in the high temperature plot than in the low temperature one.

The number of leaves on the main stalk was not always constant, but the high temperature plot was about one leaf smaller in number of leaves than the low temperature one.

Height of plant.—Figure 4 shows that the height of plant grown in the high temperature plot was very taller than that of the low temperature one.

Length and width of blade.—As shown in Figure 5, the length of blade was longer in the high temperature plot than that of the low temperature one. On the contrary, the blade was wider in the low temperature plot than in the high temperature one.

Percentage of clinging portion of leaf.—As shown in Table 2, the percentage of clinging portion of leaf was markedly higher in the high temperature plot than in the low temperature one at the same stage of growth.

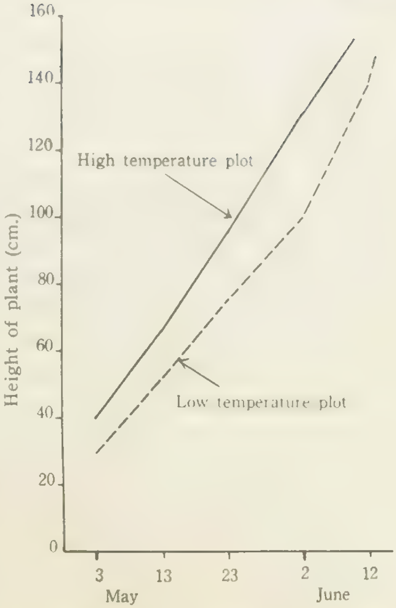


Fig. 4. Effect of temperature on the height of corn plant.

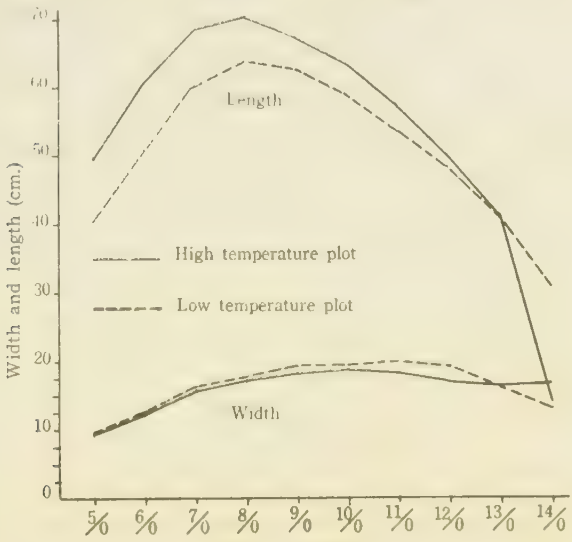


Fig. 5. Effect of temperature on the length and width of blade of corn.

Table 2. Effect of temperature on the clinging portion of corn leaf at the harvest time.*

Temperature treatment	Stage of growth					
	2/0	3/0	4/0	5/0	6/0	7/0
High temperature	100**	86	42	20	18	0
Low temperature	50	39	10	0	0	0

* Each value indicates an average of 10 plants.
** 100 presents complete clinging of leaf.

Proportion of each internode length to total internode length.—Table 3 shows that the percentage of each internode length to total internode length was higher in the high temperature plot than in the low temperature plot from 4th to 7th internode, whereas in the internodes above 7th internode reverse relation was shown.

Table 3. Effect of temperature on the proportion of each internode length to total internode length (at 2 days after the beginning to shed pollen, in per cent).*

Temperature treatment	Order of internode												
	3	4	5	6	7	8	9	10	11	12	13	14	15
High temperature	0.3	4.0	6.8	10.1	11.4	10.1	10.1	9.3	9.2	7.0	5.9	5.8	9.8
Low temperature	0.3	2.4	5.3	8.8	9.9	11.3	11.5	9.8	9.7	8.2	6.7	6.4	9.6

* Each value indicates an average of 10 plants.

Green and air dry weights.—The green and air dry weights of leaf, stalk, tassel and ear are given in Table 4. Data in this table are average of 5 pots, and each pot contains 2 plants. The average weights of the green and air dry leaf, stalk, tassel and ear produced in the high temperature plot were much less than those produced in the low temperature one.

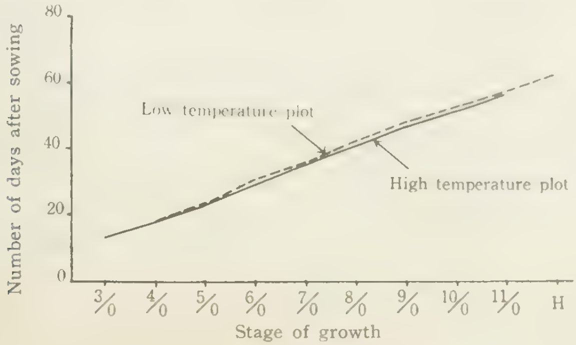
Table 4. Effect of temperature on the green and air dry weights of corn plant (in grams per pot of 2 plants).

Temperature treatment	Green weight*					Air dry weight*				
	Leaf	Stalk	Tassel	Ear	Whole plant	Leaf	Stalk	Tassel	Ear	Whole plant
High temperature	191.6	145.1	19.1	12.4	368.2	29.2	14.6	4.8	1.2	49.8
Low temperature	209.9	179.6	26.4	15.9	431.8	33.7	20.6	7.4	1.7	63.4

* Each value indicates an average of 5 pots.

b. Oats.

Leaf emerging stage.—As shown in Figure 6, the plant grown in the high temperature plot was a little faster in the appearance of leaves



H = Beginning to head

Fig. 6. Effect of temperature on the appearance of leaves on the main stalk of oats.

on the main culm than the plant grown in the low temperature one and the leaves on the main culm were about 1 smaller in number in the former than in the latter.

The heading of each culm in the high temperature plot hesitated and the heads did not completely emerge from the sheaths of top leaves and most of them stayed within the sheaths.

Height of plant.—Figure 7 shows that the height of plant grown in the high temperature plot was taller than that of the low temperature one.

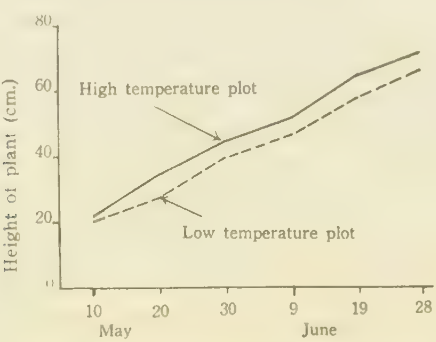


Fig. 7. Effect of temperature on the height of oats plant.

Length and width of blade.—As shown in Table 5, the length of blade was longer in the high temperature plot than in the low temperature one. On the contrary, but blade was wider in the low temperature plot than in the high temperature plot.

Table 5. Effect of temperature on the length and width of blade on the main culm of oats (in cm.).

Temperature treatment	Length*			Width*		
	6/0	7/0	8/0	6/0	7/0	8/0
High temperature	33.7	36.4	31.1	0.9	1.2	1.3
Low temperature	31.7	32.7	27.2	1.1	1.3	1.4

* Each value indicates an average of 25 plants.

Percentage of clinging portion of leaf.—Percentage of clinging portion of leaf from each plot is given in Table 6. The percentage of clinging portion of leaf was markedly higher in the high temperature plot than in the low temperature one at the same stage of growth.

Table 6. Effect of temperature on the clinging portion of oats leaf at the harvest time.*

Temperature treatment	Stage of growth				
	4/0	5/0	6/0	7/0	8/0
High temperature	100**	95	39	13	0
Low temperature	100	62	27	0	0

* Each value indicates an average of 25 plants.
** 100 presents complete clinging of leaf.

Number of tillers.—Table 7 shows that the plant grown under the high temperature condition was larger in number of head-producing tillers than that grown under the low temperature one and there was the reverse relation in no head-producing tillers.

Table 7. Effect of temperature on the number of tillers of oats.

Temperature treatment	Total number* of tillers	Number of head-* producing tillers	Number of no * head-producing tillers
High temperature	30.6	12.6	17.8
Low temperature	29.6	10.4	19.2

* Each value indicates an average of 5 pots.

Green and air dry weights.—The green and air dry weights of main culm and tiller in grams per pot of 5 plants are given in Table 8. Data in this table are average of 5 pots. The average weight of main culm and tiller produced under the high temperature condition was greater than those produced under the low temperature condition.

Table 8. Effect of temperature on the green and air dry weights of oats (in grams per pot of 5 plants).

Temperature treatment	Green weight*			Air dry weight*		
	Main culm	Tiller	Whole plant	Main culm	Tiller	Whole plant
High temperature	42.5	51.1	93.6	10.5	9.0	19.5
Low temperature	42.2	45.1	87.3	10.1	8.2	18.3

* Each value indicates an average of 5 pots.

c. Red clover.

Growth.—The growth of stem was faster at the beginning of treatment, the growth type was more erect, the flowering time was earlier and the whole plant was shorter in the high temperature plot than in the low temperature one. The petiole of plant grown under the high temperature condition remarkably elongated and the leaf of this plant was paler green color than that grown under the low temperature condition.

As shown in Table 9, the length of longest stem and the total length of branches of red clover stem grown under the low temperature condition were longer than those grown under the high temperature one at the harvest time. The branches of stem were larger in number in the former than in the latter.

Table 9. Effect of temperature on the growth of red clover.

Temperature treatment	Length of longest stem*			Total number of branches of stem (at harvest time)*	Total length of branches of stem (at harvest time)*
	May 8	May 23	June 15		
	cm.	cm.	cm.		cm.
High temperature	4.8	19.6	61.7	25.5	529.0
Low temperature	2.8	10.6	71.3	39.0	708.3

* Each value indicates an average of 15 plants.

Green and air dry weights.—The green and air dry weights of leaf, stem and flower head in grams per pot of 3 plants are given Table 10. Data in this table are average of 5 pots. The average

weights of the green and air dry leaf, stem and flower head produced in the high temperature plot were much less than were those in the low temperature one.

Table 10. Effect of temperature on the green and air dry weights of red clover (in grams per pot of 3 plants).

Temperature treatment	Green weight*				Air dry weight*			
	Leaf	Stem	Flower head	Whole plant	Leaf	Stem	Flower head	Whole plant
High temperature	92.3	66.7	17.3	176.3	19.1	15.3	3.5	37.9
Low temperature	176.0	112.9	22.6	311.5	35.4	25.7	4.5	65.6

Each value indicates an average of 5 pots.

3. Chemical composition

a. Corn.

The results of chemical analysis of corn fodder are given in Table 11.

Crude protein.—Lower leaf of corn was higher in crude protein content than upper one. This trend was more pronounced in the high temperature plot than in the low temperature one. Each leaf from the high temperature plot was markedly higher in crude protein content than that from the low temperature one. The crude protein contents of stalk and tassel were also markedly higher in the high temperature plot than in the low temperature one.

Crude fiber.—In this study, the crude fiber content of corn leaf did not show marked difference in any stage of growth in the two plots. However, the content of leaf was remarkably higher in the high temperature plot than in the low one. The stalk grown under the high temperature condition was also higher in the crude fiber content than that grown under the low temperature one, whereas the content of tassel did not show difference between the two plots.

Ether extract.—The ether extract content of corn leaf did not any difference in any stage of growth between the two plots, while the contents of stalk and tassel were higher in the high temperature plot than in the low one.

Nitrogen-free extract.—The nitrogen-free extract of corn leaf was markedly higher in the low temperature plot than in the high temperature one.

Crude ash.—Temperature did not affect the crude ash content of corn leaf. The stalk and tassel from the high temperature plot were

Table 11. Effect of temperature on the chemical composition of corn fodder. Data expressed in percentage of dry weight.

Plant organ	Crude protein		Crude fiber		Ether extract		N-free extract		Crude ash		Lignin		Crude starch		Reducing sugar		Sucrose	
	H.T.	L.T.	H.T.	L.T.	H.T.	L.T.	H.T.	L.T.	H.T.	L.T.	H.T.	L.T.	H.T.	L.T.	H.T.	L.T.	H.T.	L.T.
Leaf, Order of leaf 5/0, 6/0	14.25	11.56	29.08	25.34	3.55	3.81	41.20	46.35	13.11	13.11	3.49	2.44	—	—	—	—	—	—
Leaf 7/0	14.56	12.13	28.63	26.43					10.87	11.11								
Leaf 8/0	15.06	12.44	29.03	27.22	3.71	2.61	42.55	48.06	9.97	10.22	3.59	3.14	—	—	—	—	—	—
Leaf 9/0	14.38	12.00	29.88	27.53					9.12	9.25								
Leaf 10/0	13.94	11.88	29.69	26.06	3.70	3.74	44.72	50.33	8.16	8.31	3.52	3.32	27.68	33.29	3.07	8.34	1.01	1.47
Leaf 11/0	13.88	11.00	29.90	27.13					7.60	7.48								
Leaf 12/0	12.94	11.06	30.11	27.34	3.78	3.67	47.62	51.20	6.95	7.07	3.38	3.18	—	—	—	—	—	—
Leaf 13/0	12.31	10.88	28.51	27.49					6.38	6.43								
Leaf 14/0, 15/0, 16/0	11.56	10.56	27.68	26.36	2.76	3.35	42.51	54.26	5.49	5.47	2.84	2.65	—	—	—	—	—	—
Stalk	7.81	5.00	29.64	26.77	3.16	2.59	49.91	58.26	9.48	7.38	5.65	4.35	29.38	37.88	10.36	20.66	1.99	3.19
Tassel	21.25	16.63	18.92	19.15	5.60	3.95	49.28	56.02	4.95	4.25	—	—	—	—	—	—	—	—

H.T. = High temperature plot. L.T. = Low temperature plot.

higher in the crude ash content than those from the low temperature one.

Lignin.—The lignin contents of stalk and leaf were higher in the high temperature plot than in the low temperature one.

Reducing sugar, sucrose and crude starch.—The reducing sugar (calculated as glucose) and sucrose contents of leaf and stalk were remarkably higher in the low temperature plot than in the high temperature one. The leaf and stalk of corn from the low temperature plot were higher in the crude starch content than those from the high temperature one.

b. Oats.

The results of chemical analysis of oats hay are given in Table 12.

Crude protein.—The leaf, stem and head of main culm and all tillers of oats were higher in the crude protein content in the high temperature plot than in the low temperature one.

Crude fiber.—The leaf and stem of main culm and tiller did not show difference in the crude fiber content between the two plots, while the content of head was lower in the low temperature plot than in the high temperature one.

Ether extract.—The ether extract contents of leaf and head of main culm and all tillers from the high temperature plot were a little higher than those from the low temperature one.

Nitrogen-free extract.—The all organs of oats produced in the low temperature plot were higher in the nitrogen-free extract content than those produced in the high temperature one.

Crude ash.—The all organs of oats grown under the high temperature condition were higher in the crude ash content than those grown under the low temperature one.

Lignin.—The lignin content of stem of main culm produced in the high temperature plot was a little higher than that produced in the low temperature plot.

Reducing sugar, sucrose and crude starch.—The leaf and stem of main culm grown under the low temperatnre condition were higher in the crude starch, reducing sugar and sucrose than those grown under the high temperature condition.

c. Red clover.

The results of chemical analysis of red clover hay are given in Table 13.

Crude protein.—The crude protein content of red clover leaf was higher in the high temperature plot than in the low temperature one, the stem did not show difference in the crude protein content between the two plots.

Table 12. Effect of temperature on the chemical composition of oats hay.

Data expressed in percentage of dry weight.

	Plant organ	Crude protein		Crude fiber		Ether extract		N free extract		Crude ash		Lignin		Crude starch		Reducing sugar		Sucrose	
		H.T.	L.T.	H.T.	L.T.	H.T.	L.T.	H.T.	L.T.	H.T.	L.T.	H.T.	L.T.	H.T.	L.T.	H.T.	L.T.	H.T.	L.T.
Main culm	Leaf	11.50	8.81	28.98	29.21	4.38	3.82	42.34	46.34	12.80	11.82	3.27	3.52	26.19	29.61	4.34	4.90	2.43	4.22
	Stem	4.81	3.81	36.88	37.25	1.20	1.22	47.11	48.21	10.00	9.51	9.66	9.01	28.85	31.02	4.56	5.88	4.10	5.32
	Head	12.25	10.50	26.50	24.84	2.62	2.13	53.58	56.22	5.06	4.91	—	—	—	—	—	—	—	—
	Tiller	11.00	9.06	26.40	26.97	4.99	4.36	45.49	47.73	12.12	11.88	—	—	—	—	—	—	—	—

H.T.=High temperature plot. L.T.=Low temperature plot.

Table 13. Effect of temperature on the chemical composition of red clover hay.

Data expressed in percentage of dry weight.

Plant organ	Crude protein		Crude fiber		Ether extract		N-free extract		Crude ash		Lignin		Crude starch		Reducing sugar		Sucrose	
	H.T.	L.T.	H.T.	L.T.	H.T.	L.T.	H.T.	L.T.	H.T.	L.T.	H.T.	L.T.	H.T.	L.T.	H.T.	L.T.	H.T.	L.T.
Leaf	17.22	15.83	18.85	18.48	5.85	5.97	46.62	48.92	11.47	10.80	2.24	2.22	19.35	19.79	2.26	3.78	0.25	0.37
Stem	6.02	6.31	31.82	31.23	2.57	2.51	52.25	53.85	7.34	6.11	5.97	5.13	33.85	34.65	7.35	8.93	1.43	2.61

H.T.=High temperature plot. L.T.=Low temperature plot.

Crude fiber.—The temperature did not affect the crude fiber contents of red clover leaf and stem.

Ether extract.—There is no difference in the ether extract content of red clover between the high and low temperature plots.

Nitrogen-free extract.—The nitrogen-free extract contents of leaf and stem were somewhat higher in the low temperature plot than in the high temperature one.

Crude ash.—The leaf and stem of red clover grown under the high temperature condition were higher in the crude ash content than those grown under the low temperature one.

Lignin.—The lignin content of red clover leaf did not show any difference between the high and low temperature plots, while the content of stem was higher in the high temperature plot than in the low temperature one.

Reducing sugar, sucrose and crude starch.—The reducing sugar, sucrose and crude starch contents of leaf and stem of red clover grown under the low temperature condition were higher than those grown under the high temperature condition.

Discussion

This experiment was conducted under the glasshouse conditions during relatively warm season, therefore it is necessary to consider that the temperatures of this experiment were extraordinarily higher than those of natural conditions in Kyūshū. The results of this experiment must be discussed under this consideration.

In general, it seems that the high temperature fastened the pace of growth of the three crops. For corn and oats, the high temperature in this experiment appeared to be more favorable for the growth of plant height than did the low temperature, while this temperature response was reverse in red clover. The result of experiment with corn agrees with the author's previous report.

The high temperature promoted the elongation of lower internodes and the low temperature promoted the elongation of higher internodes of corn. The elongation of lower internodes probably partly accounts for the lodging of corn plant.

The low temperature was more favorable for the maintenance of greeness of corn and oats leaves than did the high temperature, and the maintenance of leaf greeness perhaps is important for forage crops.

It seems that the extraordinarily high temperature interfered with smooth emergence of oats head, and the blade length of corn and oats was longer in the high temperature plot than in the low temperature one and the blade width of the two crops was larger in the latter than

in the former. Red clover showed the small type of growth under the high temperature condition. These fact indicates the extraordinarily high temperature was probably unfavorable for normal growth of the three crops.

Under the conditions of this experiment, for corn and red clover the low temperature appeared to be more favorable for the production of fresh and air dry herbages than did the high temperature, whereas in oats the reverse relation was observed.

The crude protein contents of corn, oats and red clover grown under the high temperature condition were higher than those grown under the low temperature one. According to Brown (1937), the percentage of crude protein declined slightly in Kentucky and Canada bluegrass and orchard grass as the temperature increased from 40° to 60°F. and then increased slightly as the temperature rose above the optimum for growth. The author's result was obtained under extraordinarily higher temperature than Brown's experiment, therefore it seems that the extraordinarily high temperature increased the protein contents of corn, oats and red clover. This result agrees with that the result of Brown's experiment which was carried out under the higher temperature condition and that of the experiment with red clover by Delwiche and Tottingham (1930).

However, the crude fiber content of leaf and stalk of corn grown under the high temperature condition was a little higher than that of the low temperature one, and the lignin contents of corn and red clover were higher under the former than under the latter.

The unfavorable effect of the high temperature on the crude fiber and lignin contents of corn, oats and red clover canceled the favorable effect of the high temperature on the crude protein contents of these three crops as forage crops.

The crude starch, reducing sugar and sucrose contents of corn, oats and red clover grown under the low temperature condition were much higher than those of the high temperature one, and those results agree with those of Brown's experiment with Kentucky and Canada bluegrass, orchard grass and Bermuda grass and those of the experiments with the other crops by the other investigators.

The following observation seems justified under the conditions of the experiment.

The extraordinarily high temperature increased the protein content and the proportion of skeletal material in corn, oats and red clover, and the low temperature increased the carbohydrate content. It seems reasonable to conclude that the extraordinarily high temperature is an unfavorable factor for the palatability of fodder corn, oats and red clover hay as the sugar content accounts for the palatability of forage

crops, and the responsibility of growth and chemical composition to temperature depends on the species of forage crops.

Summary

1. A partial review of literature dealing with the effect of temperature on the growth and chemical composition of forage and the other crops is presented.
2. Corn, oats and red clover were grown in glasshouses and the effect of different temperature treatments on their growth and chemical composition was determined. The temperatures used in this experiment were extraordinarily higher than those in the field conditions.
3. A high temperature fastened the appearance of leaf on the main stalk of corn and leaf on the main culm of oats, tasseling of corn and heading of oats.

   The height of corn and oats was taller in the high temperature plot than in the low temperature one. The flowering time of red clover was fastened in the high temperature plot, while the growth type was shorter in the high temperature plot than in the low temperature one.
4. The percentage of each internode length to total internode length of corn was higher in the high temperature plot than in the low temperature one from 4th to 7th internode, whereas in the internodes above 7th internode reverse relation was shown.
5. The percentage of clinging portion of corn and oats leaf grown under the high temperature condition was larger than that grown under the low temperature one.
6. It seems that the extraordinarily high temperature interfered with smooth emergence of oats head. The blade length of corn and oats was larger in the high temperature plot than in the low temperature one, while the blade width was larger in the latter than in the former.
7. In this experiment, average green and air dry weights of each organ of corn and red clover produced in the high temperature plot were much less than those produced in the low temperature one, whereas there was the reverse relation in oats.
8. Lower leaf of corn was higher in the crude protein content than upper one. This trend was more pronounced in the high temperature plot than in the low temperature one. Each organ of three crops grown under the high temperature condition was higher in the crude protein content than those grown under the low temperature one with the exception of red clover stem.
9. The crude fiber content of leaf and stalk of corn and head of oats

grown under the high temperature condition was higher than those grown under the low temperature one.

10. Each organ of oats and red clover did not show difference in the crude fiber content between the two plots.
11. The ether extract content of corn stalk and tassel and all organs of oats was higher in the high temperature plot than in the low one.
12. The nitrogen-free extract content of corn leaf, all organs of oats and red clover was higher in the low temperature plot than in the high temperature one.
13. The stalk and tassel of corn and all organs of oats and red clover grown under the high temperature condition were higher in the crude ash content than those grown under the low temperature one.
14. The lignin content of corn stalk and leaf and red clover stem was higher in the high temperature plot than in the low one.
15. The leaf and stalk of corn, leaf and main culm of oats and red clover leaf and stem grown under the low temperature condition were higher in the reducing sugar, sucrose and crude starch than those grown under the high temperature condition.

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Studies on the denaturation of proteins with sodium-p-aminosalicylate (PAS)

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INTRODUCTION

It has been reported by Yoshinari[109] that the prevention of blood coagulation with PAS was due to the inhibition of fibrin formation. This is because PAS dissolves profibrin. Besides profibrin, however, almost all kinds of proteins are very easily dissolved with PAS. The relationship of the chemical structure of PAS to that of amide compounds suggested that the polarity of the PAS molecule accounts for the dissolving of proteins.

PAS is considered to have two actions in dissolving proteins. First, this compound, like glycine,[40] increases the dielectric constant of the solvent. If this is actually true, proteins in the PAS solution would not undergo denaturation. Second, PAS, like urea, acetamide, and guanidine, breaks down hydrogen bonds between peptide chains and is bound to the liberated peptide linkages.[93] If the latter is the main reason for the dissolving of proteins, proteins can be expected to be denatured by the dissociation or unfolding of their peptide chains. It is also probable that the disaggregation of proteins[12-15, 34, 35, 48, 63, 76, 83, 93, 98, 105, 108] would take place in the concentrated PAS solution the same as did in the concentrated urea solution.

To explain the mode of the dissolving action of PAS and elucidate the mechanism of denaturation, if denaturation occurred, the author estimated the sedimentation constants, the diffusion constants, and the intrinsic viscosities of serum albumin, ovalbumin, and hemoglobin in concentrated PAS solution, comparing these with those of native proteins. The molecular weights, and the frictional and axial ratios

of the above mentioned proteins were determined from these measurements.

From these data it has been concluded that proteins appeared to undergo denaturation in concentrated PAS solution. The binding of PAS by protein was also assumed to play the most important role in the PAS-denaturation. This was ascertained by measuring the amounts of PAS bound by proteins, and by the properties of the monolayer film formed with protein denatured by PAS.

The conception of the PAS-denaturation mechanism was discussed by studying the optical rotations of denatured proteins, and by considering the ionization of PAS and protein.

Experimental

Experimental Materials

Proteins:

Serum albumin (horse): Crystallized from fresh horse blood by the method of McMeekin.[44, 69, 107]

Ovalbumin (chicken): Crystallized from fresh eggs by the method of Sörensen and Höyrup.[55, 61, 92]

Hemoglobin (horse): Crystallized from horse red corpuscles by the usual method.

These proteins were lyophilized by the freeze-dry method after electrodialysis. Examination by ultracentrifugation and electrophoresis revealed that they were homogeneous.

Sodium-p-Aminosalicylate (PAS):

Commercial PAS was twice recrystallized. m.p. 112°C. (decomp.)

Experimental Methods

1. The estimation of the sedimentation constants[67, 71]

Using Spinco's Model E Ultracentrifuge, the sedimentation constants of the proteins were estimated from the rate of sedimentation in phosphate solution containing PAS (in this article the proteins dissolved in this solution were represented as "PAS treated or denatured proteins"), and in a solution of 0.05 M phosphate and 0.2 M NaCl (as "native proteins").

The values of st of serum albumin and hemoglobin were calculated by the usual equation;

$$\mathrm{st} = \frac{x_n - x_{n-1}}{\left(\frac{x_n + x_{n-1}}{2}\right) w^2 \Delta t}$$

where x is the distance from the axis of rotation and x_n and x_{n-1} refer to positions of boundaries at time t_n and t_{n-1}, w is the rotational speed in radians per second and Δt is the difference between t_n and t_{n-1} in seconds.

Since the boundary of ovalbumin in the PAS solution was not obtainable by the above method, the value of st of ovalbumin was calculated by the methods of Baldwin,[7] and of Gutfreund and Ogston.[37] The value of st by Baldwin's method was obtained by the following equation.

$$\mathrm{st}=\frac{2.303}{2w^2\cdot t}\left\{-\log\left(1-\frac{x\sum_i (x_i-x_0)^2\left(\frac{dn}{dx}\right)_i+2x_0\,\Delta x\sum_i (x_i-x_0)\left(\frac{dn}{dx}\right)_i}{N_0\,x_0^2}\right)\right\}$$

where $\quad N_0=\dfrac{c_0\,M^2\,m_1\,m_2\,LH\,\Delta n}{\tan\,\theta}\left(\dfrac{x_0}{x}\right)^2$, c_0 is the initial concentration of protein, M is the degree of magnification of the enlarged photograph of the boundary, m_1 is the degree of magnification of the camera lens; 2.17, m_2 is that of cylindrical lens; 3.49, L is the length of the optical arm; 58.1 cm, H is the thickness of the cell (cm), θ is the magnitude of the bar angle, and Δn is the refraction increment; 0.00186. x, t, w are given as the same as mentioned above.

The value of st was also obtained by the following equation, given by Gutfreund and Ogston,

$$\mathrm{st}=\frac{2.303}{2w^2}\cdot b$$

where b is given from the equation;

$$b=-\log\left\{1-\frac{2\,Q't}{c_0\,x_0^2}\right\}\frac{1}{t}$$

and

$$Q'=\int_x^x x\int_x^x x\,\frac{dc}{dx}\,dx\;dx$$

moreover

$$\int_x^x\frac{dc}{dx}\,dx=\frac{1}{K}\int_x^x\frac{dn}{dx}\,dx$$

Here, K is 12.68.

The correction of st for the concentration of protein was carried out as follows. The corrected value s_0 is given by st $(1+kc)$, where k is obtained from the values of intrinsic viscosities in any case where k is more or less than 8.

$$k = 0.75\ [\eta]+5 \qquad \text{where} \quad k<8$$
$$k = [\eta]+5.5 \qquad \text{where} \quad k>8$$

After the corrections were made for temperature and the influence of the solvent, the sedimentation constant, s_{20w}, was obtained by the following equation;

$$s_{20w} = s_0 \frac{\eta_t}{\eta_{tw}} \cdot \frac{\eta_{tw}}{\eta_{20w}} \cdot \frac{(1-V_{20}\,\rho_{20w})}{(1-V_t\rho_t)}$$

where the value of V_t is given as 0.75.

2. The estimation of the diffusion constants[1, 18, 33, 42, 54, 60, 79, 80, 102, 103]

Using Neurath's type of cell,[75] the diffusion constants of the proteins were estimated from the photograph of the diffusion boundary obtained by Schlielen's cylindrical lens method.[65, 98] The apparent value of the diffusion constants of proteins were calculated by the maximum ordinate method, using following equation;

$$D\mu = \frac{\mu^2}{2t} \cdot F$$

by the maximum ordinate-area method using

$$D_A = \frac{A^2}{4\pi t\, H_m{}^2} \cdot F$$

and by the moment method using

$$D_M = \frac{\sigma^2}{2t} \cdot F$$

where the area under the diffusion curve, A, is given by $N \cdot w$, μ is half the distance between both inflexion points of the curve whose height, $H\mu$, is given $H\mu = \frac{H_m}{\sqrt{e}}$, H_m is the height of the peak, and σ is the standard deviation of the curve.

The mean value of these apparent diffusion constants, D_m, was obtained. After correcting for temperature by using the equation below,[2] the corrected value, D_0', was obtained:

$$D_0' = D_m + \frac{A\ M_0}{4\ H_m\ t} \cdot F$$

The zero time correction was made by Longsworth's equation:[67]

$$D_0' = D_0(1+\Delta t/t)$$

where D_0 is the corrected value for the diffusion constant and Δt is a small increment added to the observed time, t, to correct the actual occurrence of the boundary. Thus the diffusion constant under standard conditions was obtained by the equation:

$$D_{20w} = D_0' \frac{\eta_{tsol}}{\eta_{tw}} \cdot \frac{\eta_{tw}}{\eta_{20w}} \cdot \frac{293}{273+\theta}$$

3. The viscosity measurements

By the usual method, using Ostwald's viscosimeter, the specific viscosities of the proteins in the concentrated PAS solution were determined. The intrinsic viscosities were calculated from the following equation

$$[\eta]=\lim_{c\to 0}\ (\eta_{sp}/c)$$

4. The molecular weights[95)]

The molecular weights of the PAS treated proteins were calculated from the equation below ;

$$M=\frac{R\,T\,s}{(1-V\rho)D}=2.44\cdot 10^{10}\cdot\frac{s_{20w}}{(1-0.9982\ V_{20})\ D_{20w}}$$

where M is the molecular weight, R is gas constant, V is specific volume of protein, ρ is the density of the solvent, s and D represent sedimentation and diffusion constant respectively.

5. The frictional ratios[74)]

Using the determined values of s_{20w}, D_{20w}, and M, the frictional ratios of the denatured proteins, f/f_0, were calculated by the following equations :

$$f/f_0=\frac{1}{6\pi\eta}\left(\frac{RT}{DN}\right)^{2/3}\left[\frac{4\,(1-V)}{3Vs}\right]^{1/3}=1.00\cdot 10^{-8}[(1-0.9982\,V_{20})/D^2_{20w}s_{20w}V_{20}]^{1/3}$$

$$f/f_0=\frac{1-V\rho}{6\pi\eta s}\left(\frac{4\pi M^2}{3VN^2}\right)^{1/3}=1.19\cdot 10^{-15}[M^{2/3}(1-0.9982\,V_{20})]/s_{20w}V_{20}^{1/3}$$

$$f/f_0=\frac{R\,T}{6\pi\eta ND}\left(\frac{4N}{3VM}\right)^{1/3}=\frac{2.89\cdot 10^{-5}}{D_{20w}(V_{20}M)^{1/3}}$$

where V_{20} is given as 0.75.

6. The axial ratios from the frictional ratios[43,73,77,81,85,97)]

The values of the axial ratios of the denatured proteins, p, were calculated from the frictional ratios of the proteins.

$$f/f_e=\frac{(1-p^2)^{1/2}}{p^{2/3}\ln\dfrac{1+(1-p^2)^{1/2}}{p}}\qquad p=\frac{b}{a}<1\ \text{for prolate}$$

$$f/f_e=\frac{(p^2-1)^{1/2}}{p^{2/3}\ \tan^{-1}(p^2-1)^{1/2}}\qquad p=\frac{b}{a}>1\ \text{for oblate}$$

where $\quad f/f_e=(f/f_0)/(1+w/V\rho)^{1/3}$

7. The axial ratios from the intrinsic viscosities [62, 70, 78, 90)]

Calculations from the values of the axial ratios of PAS denatured proteins were made from the values of the intrinsic viscosities of the proteins by the following equations :

$$\nu = \frac{\left(\frac{1}{p}\right)^2}{15\left[\ln\left(\frac{2}{p}\right)-\frac{3}{2}\right]} + \frac{\left(\frac{1}{p}\right)^2}{5\left[\ln\left(\frac{2}{p}\right)-\frac{1}{2}\right]} + \frac{11}{15} \qquad p=\frac{b}{a}\ll 1 \text{ for prolate}$$

$$\nu = \frac{16}{15}\,\frac{p}{\operatorname{arc\,tan} p} \qquad p=\frac{b}{a}\ll 1 \text{ for oblate}$$

where $\nu=\nu'/\left(1+\frac{w}{V\rho}\right)$ and $\nu'=100[\eta]/V$

8. The measurements of the optical rotations

By Goerz Lippich's polarimeter, the specific rotations of the proteins were measured by using a sodium lamp, manufactured by Toshiba, as a light source. The accuracy of the measurements was $\pm 0.01^\circ$.

9. The measurements of the bound PAS by proteins[56-60]

The amounts of PAS bound by protein were measured by dialysis experiments after Klotz, using collodion bags which were prepared by collodion-ether solution. The collodion bags were filled with 10 cc of protein solution usually near 0.5% concentration. The bag was immersed in 20 cc of solution of the 0.05 M phosphate buffer or 0.1 M carbonate buffer solution of different pH's, containing different concentration of PAS, and placed in a cold room at approximately 5°C for a period of 5 to 7 days, an interval sufficient for the attainment of equilibrium. The bag was then removed and the external solution analysed for PAS. For each PAS concentration a control tube was also prepared, which contained only buffer solution inside the bag. The correction was made for the PAS binding by collodion membrane. About 7% of the free PAS in solution was bound by the collodion membrane.

10. The determination of PAS

The determination of PAS was carried out by the method of Tennet and Leland,[100] using Shimazu's photoelectric colorimeter with a filter of 620 mμ.

11. The formation of monolayer films of PAS-denatured ovalbumin on KCl solution[3, 10, 11, 16, 19, 20, 22, 38, 39, 41, 46, 47, 72, 99]

Ovalbumin was dissolved in the solutions containing different concentrations of PAS, 5 to 20%, and placed at room temperature, approximately 20°C, for 30 minutes. Isopropyl alcohol was added to 30% prior to spreading on a substrate. A control spreading solution of ovalbumin was prepared by dissolving it in water and adding isopropyl alcohol to 30%.

As a substrate 0.5 M KCl aq. solution of pH 5.5 was used.

Surface pressure was measured by Sasaki's modification of Wilhelmi's

hanging plate method.[87] The measurement were carried out at 21 to 24°C. The surface pressure, F, was expressed in dyne/cm and the area of the spread protein monolayer, A, in M^2/mg.

The limiting area or co-area was obtained from the compressibility, $-(1/A)(dA/dF)$, vs area curve of the monolayer film.

The molecular weights[45] of the denatured ovalbumin obtained from the FA vs F lines at different concentrations of PAS were calculated by the equation below ;

$$(FA)_{F\to 0}=\frac{10^{-3}}{M}RT$$

Results

1. The sedimentation constants of the native and the PAS treated proteins[25, 26]

The sedimentation constants of the PAS treated proteins were smaller than those of the natives, particularly in the case of hemoglobin as indicated in Table 1.

Table 1. Sedimentation constants of native and 20% PAS-treated proteins at pH 7.8.

Protein	$s_{20w} \cdot 10^{13}$		
	Ovalbumin	Serum albumin	Hemoglobin
Native	3.59	4.45	4.56
PAS-treated	2.70	3.32	2.01

2. The diffusion constants of the native and the PAS treated proteins[27]

As with the sedimentation constants, the diffusion constants of

Table 2. Diffusion constants of native and 20% PAS-treated proteins at pH 7.8.

Protein	$D_{20w} \cdot 10^{7}$		
	Ovalbumin	Serum albumin	Hemoglobin
Native	7.76[a]	6.1 – 6.5[b]	6.30[c]
PAS-denatured	5.37	4.57	5.45

a: G. Lamm, A. Polson, Biochem. J., 30, 528 (1936)

b: R. A. Kekwick, ibid., 32, 552 (1938). G. R. Cooper, H. Neurath, Chem. Revs., 30, 357 (1942). H. Neurath, G. R. Cooper, J. O. Erickson, J. Biol. Chem., 138, 411 (1941)

c: A. Tiselius, D. Gross; Kolloid Z., 66, 11 (1938)

20% PAS treated proteins at pH 7.8 were also smaller than those of the native ones as indicated in Table 2.

3. The molecular weights, frictional ratios, intrinsic viscosities, and axial ratios of the native and the PAS denatured proteins[28]

Results obtained as summarized in Table 3.

Table 3. Molecular weights, frictional ratios, intrinsic viscosities, and axial ratios of native and PAS-denatured proteins.

Proteins		M	f/f_0	$100[\eta]$	ν'	Axial ratios $w=0.3$ calculated from			
						f/f_0		ν	
						pro-late	oblate	pro-late	oblate
Ovalbumin	native[a]	45,000	1.16	4.3	5.7	3.5	3.9	3.3	4.0
	denatured	49,000	1.62	7.7	10.3	8.3	9.8	6.4	9.3
Serum albumin	native[b]	70,000	1.27	6.2	8.3	5.2	6.2	5.0	6.3
	denatured	71,000	1.69	9.3	12.4	9.4	11.4	7.4	11.6
Hemoglobin	native[c]	69,000	1.16	4 0	5.3	3.5	3.9	3.1	3.5
	denatured	36,000	1.77	10.8	14.4	10.7	13.3	8.3	13.7

a: G. Lamm, et al., Biochem. J., 30, 528 (1936). T. Svedberg, et al., J. Am. Chem. Soc., 50, 525 (1928).
b: R. A. Kekwick, Biochem. J., 32, 552 (1938). H. Neurath, et al., J. Biol. Chem., 139, 411 (1941). T. Svedberg, et al., J. Am. Chem. Soc., 50, 3318 (1928).
c: A. Tisselius, et al., Kolloid Z., 66, 11 (1938). H. L. Fevold, Adv. Protein Chem., 6, 211 (1951)

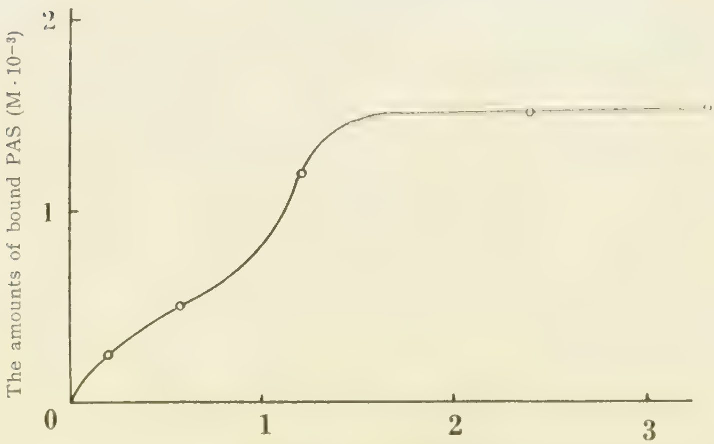

Fig. 1. The amounts of bound PAS at various concentrations of PAS.

4. The amounts of PAS bound by ovalbumin with increasing concentrations of PAS[30]

The amounts of PAS bound by ovalbumin at pH 7.4 in various concentrations of PAS of measured by the equilibrium dialysis method after Klotz. The concentration of protein inside the collodion bag was $1.11 \cdot 10^{-6}$ M/10 cc. The experiments were carried out, using 20 cc of each of the following PAS solutions as the external solutions; 1.43, 0.95, 0.48, 0.24, and 0.10 M. The results obtained are shown in Fig. 1.

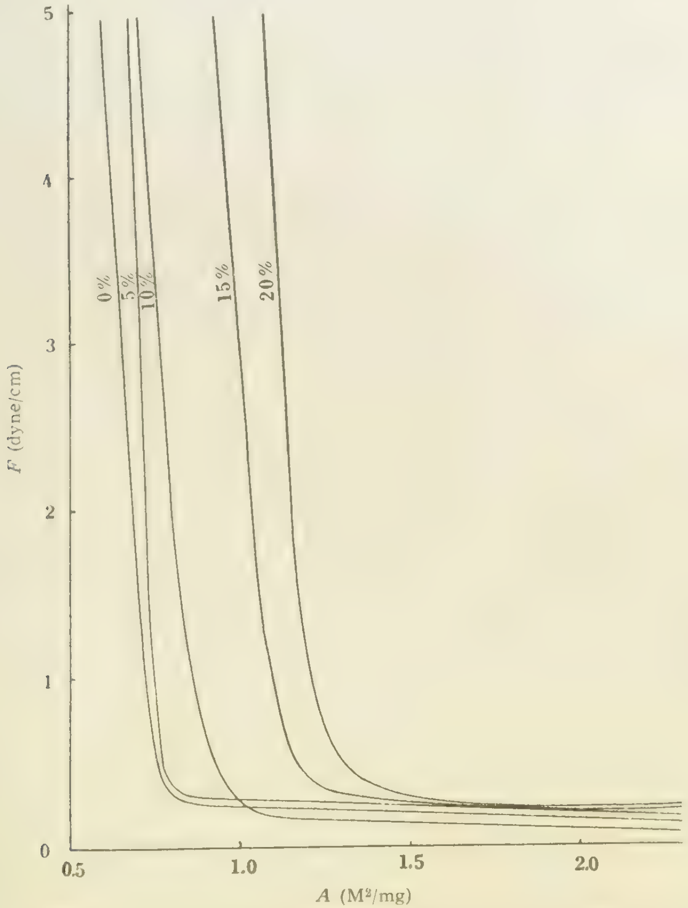

Fig. 2. The $F-A$ curves of ovalbumin monolayer films spread from 5, 10, 15, and 20% PAS solutions respectively.

5. The surface pressure vs area curves ($F-A$ curves) of PAS-treated ovalbumin[31]

The F vs A curves were obtained from the monolayers which were spread from the solutions of ovalbumin, containing 5 to 20% PAS, on 0.5 M KCl solution of pH 5.5 at 21°C as shown in Fig. 2. The surface pressure measurements started in one hour after spreading the monolayers.

6. The limiting areas of the monolayers of PAS-denatured ovalbumin[31]

The limiting areas were obtained from the curves given in Fig. 2 by the method mentioned above.

Table 4 shows the relationship between the concentrations of PAS in the solutions, in which ovalbumin was treated, and the limiting areas of the monolayer films.

Table 4. The limiting areas obtained for the monolayers of denatured ovalbumin in PAS solutions of different concentration.

Concentration of PAS %	Limiting area M^2/mg
0	0.787
5	0.812
10	0.969
15	1.157
20	1.354

7. The FA vs F curves of the monolayer films of PAS-denatured ovalbumin[31]

The values of FA were calculated from the $F-A$ curves given in Fig. 2 and plotted against the values of F, thus obtaining the straight lines for each concentration of PAS. Moreover all the lines joined at one point on the FA axis, when each was extrapolated to $F\rightarrow 0$. The value of $(Fa)_{F\rightarrow 0}$ for 1 mg protein was given as 0.053 as shown in Fig. 3.

8. The optical rotations of proteins[29]

The dependence of the specific rotations of serum albumin and ovalbumin upon the concentrations of PAS in solution is shown in Fig. 4 and 5 respectively.

The optical rotations of both proteins were measured in M/10 carbonate buffer solution of pH 10 containing PAS and in M/5 phosphate buffer solution of pH 7.7 containing PAS at 8–11°C. The concentration of serum albumin in solutions was 0.5% and that of ovalbumin was 0.4%.

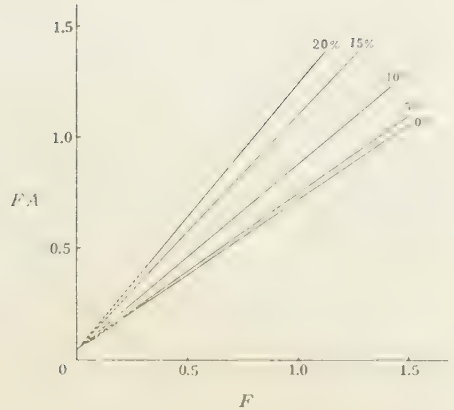

Fig. 3. The relations between FA and F values in different concentrations of PAS.

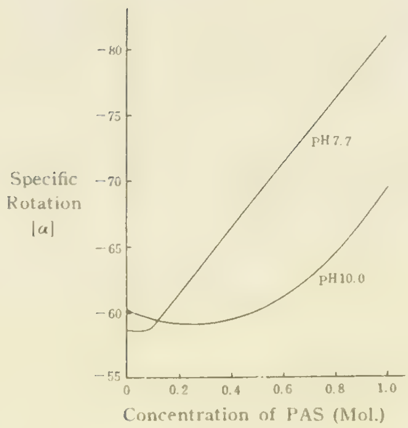

Fig. 4. The specific rotations of serum albumin at various concentrations of PAS at pH 7.7 and 10.

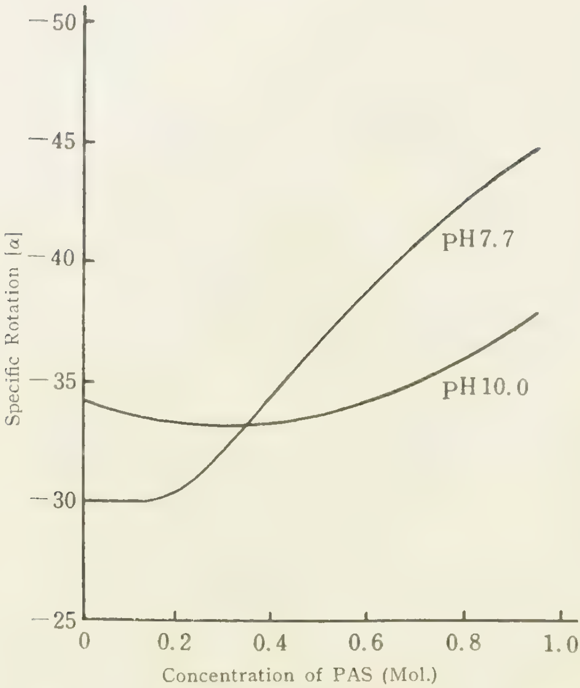

Fig. 5. The specific rotations of ovalbumin at various concentrations of PAS at pH 7.7 and 10.

The progressive changes in the specific rotations of PAS denatured serum albumin and ovalbumin at different pH's are shown in Fig. 6

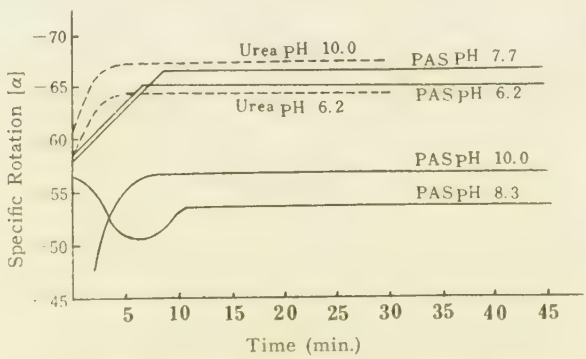

Fig. 6. The progressive changes in the optical activities of serum albumin in 10% PAS solution of different pH's at 10–11°C.

and 7 respectively. In these figures the values of the specific rotations are plotted with time measured in minutes. The measurements were carried out in 10% PAS and urea solutions of pH 6.2, 7.7, 8.3, and 10. The concentration of serum albumin in PAS- and urea solution was 0.65% and 0.8% respectively and that of ovalbumin in both solutions was 0.7%.

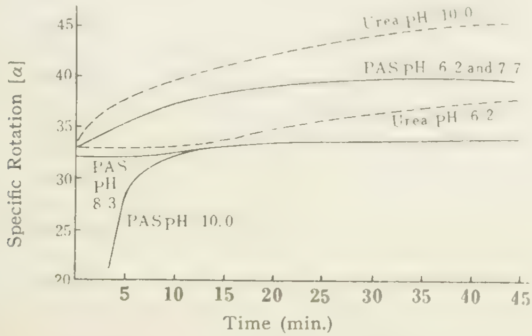

Fig. 7. The progressive changes in the optical activities of ovalbumin in 10% PAS solution of different pH's at 10–11°C.

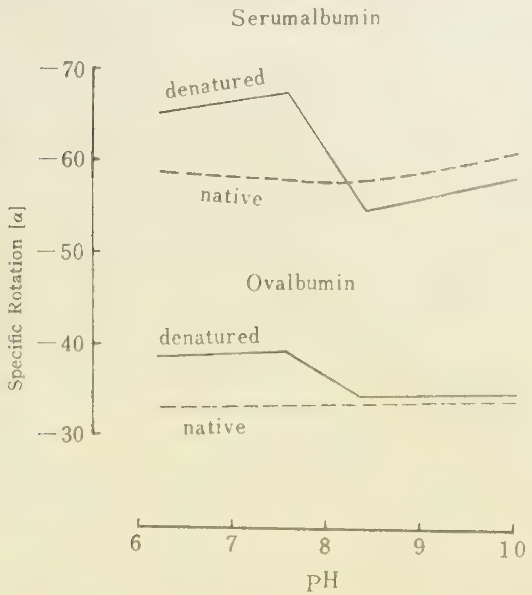

Fig. 8. The dependence of the specific rotations upon pH.

The dependence of the specific rotations of serum albumin and ovalbumin upon pH is shown in Fig. 8. The measurements were carried out in 10% PAS solutions of different pH's at 10–11°C. The concentration of serum albumin and of ovalbumin was 0.65% and 0.7% respectively. The specific rotations of native proteins, which were dissolved in buffer solutions, were also measured in contrast with those of the PAS denatured proteins.

9. Tne dependence of the amounts of bound PAS upon pH[30]

As shown in Fig. 9, the r vs pH curve gave a point of the minimum value of r, where r refers to the ratio of the moles of bound PAS to moles of total protein.

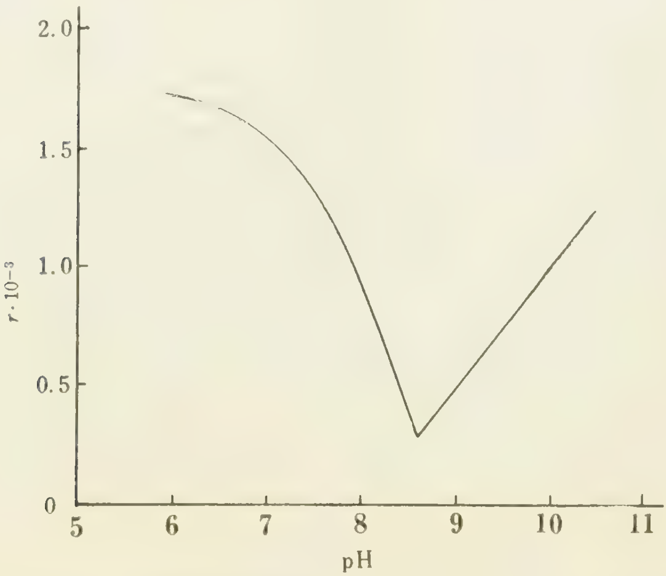

Fig. 9. The dependence of the amounts of PAS bound by ovalbumin upon pH.

Discussion and conclusion

The sedimentation constants of ovalbumin, serum albumin, and hemoglobin in concentrated PAS solutions were smaller than those of the native proteins, especially in the case of hemoglobin, for which a remarkably low value was obtained, as indicated in Table 1. The same tendency observed in the concentrated urea solution was observed in the PAS solution. This seemed to suggest that the PAS effect did

not depend only upon increasing the dielectric constant of the solvent, but also upon the change in the shape of the protein molecule.

It is well known that two factors influence the sedimentation constant of a protein -the molecular weight, and the shape or axial ratio of the molecule. Regard to the shape of the protein molecule, the increase in the asymmetry causes a decrease in the sedimentation constant, if the molecular weight is constant. For instance, although zein,[104, 108] ovalbumin,[64] and the protein of *Bac. tuberculosis*[89] have the same molecular weight, approximately 40,000, they have different values of f/f_e; 2.4, 1.16, and 1.25 and of $1/p$; 32, 3.5, and 5.0 respectively. Thus their sedimentation constants are 1.9, 3.55, and 3.4 respectively.

The values of the diffusion constants of PAS-treated proteins were also smaller than those of the native ones, as indicated in Table 2. The decrease in the diffusion constants of the proteins suggests also a decrease in their molecular weight or an increase in their degree of asymmetry. As indicated in Table 3, however, the molecular weights of ovalbumin and serum albumin were maintained constant in the concentrated PAS solution, unlike hemoglobin, which underwent dis-aggregation in the same solution. The molecular weight of the split hemoglobin molecule was one half of the native. Consequently, it was assumed that proteins in the concentrated PAS solution changed their molecular forms to those which have greater asymmetry in their molecular shape. In fact, the frictional and axial ratios, and the intrinsic viscosities of these proteins treated with PAS were greater than those of the native ones, as indicated in Table 3. These results suggested, first, that the PAS treated protein underwent denaturation, and, second, that the molecular shape of the denatured protein was oblate. It was not prolate, since, in the case of denatured proteins, the axial ratios calculated from the frictional ratios coincided with those calculated from the intrinsic viscosities when the molecular shape was assumed to be oblate. The axial ratio of the denatured serum albumin and ovalbumin, however, were never so large, e.g., only twice as large as those of the native proteins. Therefore, it was concluded that the molecules of PAS denatured proteins were slightly enlarged transversely to their longitudinal axis. Such a change in molecular shape seemed to be due not only to the entrance of PAS molecules into proteins, but also to the formation of new hydrogen bonds with proteins. This led to the enlargement of the protein molecule. The polar PAS molecules, which were driven by their thermal movements between the peptide chains, could cleave the original hydrogen bonds between the peptide chains and bind to the liberated peptide linkages by the formation of new hydrogen bonds. In this case the negatively charged groups of PAS molecules also would combine with the liberated, positively charged groups of the protein molecules. As the results

of these combinations, the protein molecule, which had partially dissociated, could again be refolded in a different manner from the original, and then would be in an enlarged or swollen state.

The assumption that the combination of PAS should occur inside the protein molecule was confirmed by the measuring the amounts of PAS bound by protein at different concentrations of PAS. As shown in Fig. 1, the binding of PAS by protein was assumed to take place in two steps depending upon the concentration of PAS. In concentrations of PAS lower than 0.01 M, the amounts of PAS bound by protein were relatively small. This seemed to suggest that PAS combined only with the surface of the protein molecule, which did not undergo denaturation. Secondly, in concentrations of PAS higher than 0.01 M, however, the amounts of PAS bound by protein increased rapidly, until it reached to the maximum value. This suggested that the binding of PAS occurred inside the protein molecule, whose folding had been disarranged by the denaturation. Thus the degree of denaturation seemed to be proportional to the amounts of PAS bound by protein.

On the other hand, the F vs A curves obtained from the spreading of monolayers of ovalbumin solutions containing 5 to 20% PAS on 0.5 M KCl solution at pH 5.5, suggested the binding of PAS occurred inside the protein molecule, as shown in Fig. 2. These curves indicated that the area of the monolayer film produced by the PAS-denatured ovalbumin increased with an increase in the concentration of PAS. At concentrations of PAS lower than 5%, the area did not increase. This same tendency was observed also in the case of limiting area of the monolayer film of the PAS-denatured ovalbumin, as shown in Table 4.

These facts seemed to suggest that in the concentrated PAS solution, the binding of PAS by protein occurred inside the protein molecule. The amounts of PAS bound by protein depended upon the concentration of PAS and upon the degree of denaturation of the protein.

Moreover, the binding of PAS by protein was assumed to occur intramolecularly and not intermolecularly. As shown in Fig. 3, all of the FA vs F lines obtained at different concentrations of PAS joined at one point (0.053) on the FA axis, when the lines were extrapolated to $F=0$. Thus the molecular weight of PAS-denatured ovalbumin corresponded to 45,000, the same as the native protein. This suggested that the number of the molecules of PAS-denatured ovalbumin in solution was just the same as that of the native protein. Therefore, it was concluded that association or aggregation of protein molecules did not occur during the denaturation.

Furthermore, the enlargement of the limiting area depended upon an increasing concentration of PAS. This suggested that the protein molecules combined more readily by hydrogen bonds with PAS than

with H_2O molecules. If the hydrogen bonds between the protein and PAS were weaker than between protein and H_2O, the PAS molecule should be removed from protein by H_2O, when the PAS-denatured ovalbumin is spread on water.

Consequently, it was concluded that the binding of PAS by a protein played the most important role in the denaturation of the protein with PAS.

In order to elucidate the dissociation and rearrangement of the denatured protein molecule, the optical rotation of the denatured proteins were studied.

It is well known that a protein solution changes its viscosity as well as its optical activity on denaturation.[4−6, 8, 9, 15, 17, 21, 23, 32, 39, 68, 82−84, 94, 101, 110] Recently, the changes in optical rotation by denaturation were studied in detail by Kauzmann[24, 51−53, 88, 91] and Jirgensons.[49,50] Small changes in the inner structure of the protein molecule, which do not give changes in the viscosity of the protein solution, can be detected by measurement of optical activities.[49] While the viscosity of a protein solution is concerned with the shape and degree of hydration or solvation of the protein molecule in solution, the optical activity of a protein solution is concerned with the particular structure of the protein molecule. The changes in optical rotation of the protein solution following changes in the inner structure of the protein molecule is very sensitive.

It was suggested by Kauzmann[51] that those substances, in which there are restricted rotations of the groups about the bonds connecting them to the asymmetric centers, possess rather large optical activities in contrast to compounds in which freedom of rotation is possible. Furthermore, the total optical rotation of a given protein molecule equals the sum of partial optical rotations of the asymmetric carbons of that protein molecule. Consequently, it is assumed that many factors affect the optical rotation of proteins; i.e., whether the peptide chains are folding or unfolding, whether the protein molecules are expanding, swelling, or contracting, etc.

From the points of view mentioned above, the changes in the optical rotation of the PAS-denatured proteins are discussed.

As shown in Fig. 4 and 5, the optical activities of serum albumin and of ovalbumin in PAS solution varied with the concentration of PAS. At pH 7.7, while the changes in the optical activities were not observed in both proteins until the concentration of PAS reached ca 0.1 M, in concentrations of PAS higher than 0.1 M, the optical activities of both proteins increased proportionately with the increase in concentration of PAS. At pH 10, however, it was observed with both proteins that the optical activities began to increase gradually after the concentration of PAS reached 0.5 M. In either case, it can be said

that the changes in the optical activities of the proteins occurred only in concentrated PAS solution, in which the amounts of PAS bound by protein were certainly large. In other words, in lower concentrations of PAS, where PAS was assumed to combine chiefly with the surface of the protein molecule without the accompaning denaturation, the optical rotation of PAS-denatured protein was almost the same as that of the native ones. However, in high concentrations of PAS, the progressive unfolding of the peptide chains was assumed to promote the binding of PAS, by the liberated peptide linkages inside the protein molecule. Thus the large optical rotations of protein was observed here, where the large amounts of PAS were assumed to combine with protein with the accompaning denaturation.

The same interpretation as above can be given to the effect of pH on the PAS denaturation, i.e., a similar relationship between the magnitude of optical activity of denatured protein and the amounts of PAS bound by protein as mentioned above is observed here.

The optical activities of the proteins, which were denatured at different pH's in 10% PAS solution for 30 minutes, are shown in Fig. 8. In a pH range lower than 8, the levorotations of PAS-denatured serum albumin and ovalbumin were larger than that of the native proteins. However, in a pH range higher than 8, the levorotation of denatured serum albumin was lower than that of the native protein, while that of the denatured ovalbumin was slightly higher than that of the native protein.

On the other hand, as shown in Fig. 9, the amounts of PAS molecule bound by proteins at a pH lower than 8 were definitely greater than those at a pH higher than 8. At a pH near 8.5 the combination of PAS and protein was at a minimum. It appeared, therefore, that in the acid range the binding of PAS by protein occurred very easily and rapidly, while in a alkaline medium it occurred slowly and with difficulty.

From these data it has been concluded that the optical activities of the denatured proteins varied with pH in the same way as did the amounts of protein bound PAS. This seems also suggest that the situation of the peptide chains of the denatured proteins is dependent upon the pH of PAS solution, in which denaturation has occurred.

This assumption was confirmed by studying the progressive changes in the optical rotations of serum albumin and ovalbumin denatured by PAS at different pH's.

As shown in Fig. 6 and 7, the optical activities of serum albumin and ovalbumin in 10% PAS solution changed with time, depending upon pH. At pH's lower than 8 in the PAS solution, the levorotations of proteins increased at the same rate as in urea solution of the same pH. The increase in levorotation of denatured serum albumin occurred

more rapidly than that of ovalbumin. However, at a pH higher than 8, for instance at pH 10, a fall in levorotations was observed in the initial stage of denaturation. Subsequently a gradual increase occurred, in the case of serum albumin, until the value of levorotation reached a level slightly smaller than that of the native protein. In the case of ovalbumin, however, the levorotation reached a slightly larger value than that of the native protein. In 10% urea solution at pH's higher than 8 levorotations of both proteins increased more rapidly than in the acid pH range, serum albumin increasing it's optical activity more rapidly than ovalbumin.

If the protein molecules simply unfolded, the ring structure would be diminished and the extent of free rotations of groups around the asymmetric carbons would increase. Thus the protein molecule would have a partially unfolded *α-helix* of peptide chains. This would cause a distinct fall in levorotation. However, a higher levorotation would be the result of the refolding of the once partially dissociated peptide chains.

Thus it was assumed that the definite fall in levorotation in the initial stage of denaturation meant that the peptide chains of the protein molecules were partially dissociated and unfolded. The gradual increase in levorotation was assumed to mean that the partially unmasked amino acid residues of the peptide chains were again bound through the PAS molecule in a relatively disordered state.

Consequently, it was concluded that during the denaturrtion of protein by PAS, the protein molecule, in any case, would be partially unfolded by PAS. At approximately the same time, PAS would combine with the liberated peptide chains, resulting in a refolding of the protein molecule. Such changes would occur in all pH ranges of the solution, but the rate of unfolding and of refolding would be different depending upon the pH of the solution. At a pH higher than 8, the refolding through PAS would occur so slowly that it could be observed. However, at a pH lower than 8, it would occur so quickly that one could not detect it.

In considering the ionization of PAS and protein at different pH's, the assumptions mentioned above appear to be more definitely confirmed.

PAS is assumed to dissociate in the following four forms as shown in Fig. 10.

In this case form 1 and 2 are not considered, since these forms will exist only in strongly acid solutions which have not been studied in these experiments. The third species is the most useful for the formation of hydrogen bond, since its $-OH$ and $-NH_2$ groups have the potential forming a hydrogen bond. PAS will exist in this form at a pH near 7. In an alkaline medium near 8.5, however, PAS

will be converted into form 4, which has only one available group, i.e., amino group, for the formation of a hydrogen bond. Here, the OH group of the PAS molecule will be ionized on the basis of the

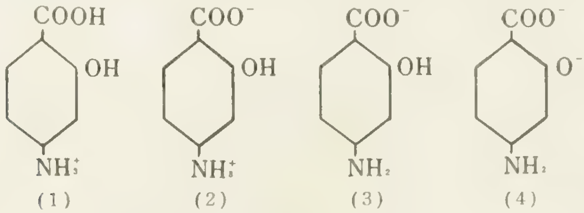

Fig. 10.

pK'value of the OH group of the PAS molecule. Moreover, the dissociation of the positively charged groups of the protein molecule is also assumed to be insufficient to combine with the negatively charged groups of PAS which are present in form 4.

Therefore, it is obvious that the binding of PAS by protein is influenced by the degree of dissociation of PAS and of protein, particularly of those groups, which have the ability to form hydrogen bonds at the lower pH's, i.e., less than 8 or 9. At pH's higher than 8, however, the groups, which have an ability to form electrostatic bonds, also influence the binding of PAS. This, in turn, controls not only the rearrangement of peptide chains during PAS denaturation but the final state of the denatured protein as well. Thus, the optical activity of the proteins is affected by the dissociation of both the PAS molecules and the proteins as a result of the influence of intramolecular binding upon the optical rotation. For instance, the facts that in the alkaline range a definite fall in the initial stages of the denaturation and then a gradual increase occurred in levorotation of PAS-denatured protein is more easily explained by the consideration of the ionization of PAS and protein as mentioned below.

In an alkaline solution PAS exists in form 4, where the NH_2 group will combine with the peptide linkages by hydrogen bonding as shown in Fig. 11.

However, the combination of O^- or COO^- groups of PAS with the positively charged groups of protein, whose dissociation is insufficient to cause electrostatic binding, will hardly occur. The smaller levorotation of the denatured protein in the initial stages will result from this. However, the electrostatic binding between the above mentioned groups will occur with the lapse of time. Thus the refolding by either increasing the linkage between petide chains by bound PAS molecules or reversal of the disordered folding of the peptide chains will occur. This will cause an increase in the levorotation to a certain extent.

Since at pH 6 to 8, where the PAS molecule exists in form 3, the binding of PAS by protein and the refolding of the dissociated peptide chains are assumed to occur rapidly in the initial stages of denaturation. The distinct fall in levorotation might not be observed under these experimental conditions, provided it could occur. The folding of the peptide chain of the denatured protein should be more complicated and disorderly than that of the native protein.

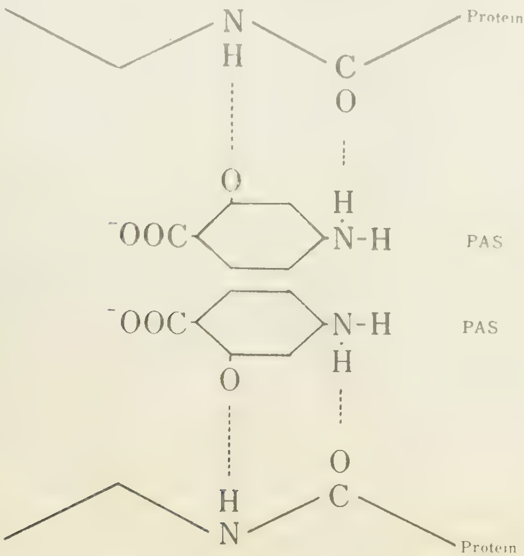

Fig. 11. The proposed scheme of the binding of PAS by protein.

The proposal of the rebinding of peptide chains through PAS seems also to be confirmed by considering the effects of sulfur-linkages upon the intramolecular changes in protein during PAS denaturation. In other words, in considering these effects of sulfur-linkages, the changes in optical activities of proteins are also clearly explained. Particularly this difference in optical activity between serum albumin and ovalbumin more clearly understood.

It is well known that the petide chains of serum albumin are folded more simply and inperfectly than that of ovalbumin.[24, 51, 52, 88, 91] The former contains many sulfur linkages, i.e., 32 half cystines in one mole compared to 2 in one more of the latter. The sulfur linkage is not considered to be cleaved during PAS denaturation.

Therefore, when the peptide chain is dissociated by denaturation, it will occur more easily with serum albumin than in ovalbumin.

However, the final extent of dissociation is greater in ovalbumin than in serum albumin, since the former contains many sulfur linkages, by which the unfolding of the peptide chains seems to be restricted to some extent. Upon refolding the dissociated peptide chains the same sulfur linkages as above will restrict the refolding, i.e., the sulfur linkages interrupt the freedom of rotation of dissociated peptide chain leading to close, disordered contact. In acid solution, as mentioned above, since unfolding would be followed rapidly by refolding, the partially dissociated peptide chains would again be bound quickly by PAS molecules. The sulfur linkage would have little effect on the changes in the folding of the molecule. However, the rearrangement of the peptide chains by PAS with the corresponding changes in optical activity of the protein would be controlled only by the inherent folding conditions of the native proteins. Consequently, serum albumin, whose peptide chains can dissociate easily, showed more rapid and larger changes in the optical activity than ovalbumin. In alkaline solution, however, the binding of PAS by protein will occur with difficulty, as mentioned above. Although dissociation of the peptide chains will be promoted by an increase in the dielectric constant of the solution with PAS, the rebinding of the partially dissociated peptide chains by the combined PAS would occur slowly. For these reasons the distinct fall in levorotation would be determined by the effect of sulfur linkages upon the refolding of the dissociated peptide chains. Although serum albumin's ability to refold is limited to an extent by its larger number of sulfur linkages, ovalbumin will be more freely and closely refolded with the resultant larger optical activity than the native protein. In alkaline solution, the smaller levorotation of denatured serum albumin compared to the native protein and the larger levorotation of denatured ovalbumin compared to its native therefor may be explained by the above consideration.

It was concluded that binding of PAS by proteins, especially binding by hydrogen bonds play the most important role in the denaturation with PAS. The sulfur linkages are of secondary importance giving a restriction in the dissociation, on the one hand, and the subsequent refolding of the peptide chains, on the other hand. In alkaline solution the electrostatic forces seem to be important for binding of PAS by protein.

Consequently, it is concluded that PAS causes the denaturation of protein molecule in the following mechanism.

When proteins are dissociated in a concentrated PAS solution, contraction of the protein molecule will occur. Then the polar PAS molecule enters the peptide chains, destroying the hydrogen bonds. Partial unfolding of the peptide chains occurs. The PAS molecules combine with the liberated peptide linkages by the formation of new

hydrogen bonds. The partially unfolded protein again fold up with PAS. The pH of the PAS solution determines the type of refolding. The refolded protein molecule is assumed to be oblate, and to be in an enlarged or swollen state.

Summary

It has been discovered that in a concentrated solution of sodium-p-aminosalicylate (PAS), protein underwent denaturation. To explain the mechanism of denaturation, the sedimentation constants, diffusion constants, and intrinsic viscosities of serum albumin, ovalbumin, and hemoglobin in concentrated PAS solution were measured and compared with those of the native proteins. The molecular weights, and the frictional and axial ratios of the above mentioned proteins were determinded from these measurements. From these results, it has been concluded that the denatured proteins had almost the same molecular weight as the native ones, except hemoglobin, which underwent disaggregation into half the native molecular weight. The shape of the denatured protein was assumed to be oblate. This suggested that the binding of PAS occurred inside the protein molecule and would cause the rearrangement of peptide chains and change in the shape of the protein. The binding of PAS to the protein molecule was ascertained by measuring the amounts of PAS bound by protein. The nature of the binding was studied by properties of the monolayer formed with protein denatured by PAS.

The changes in optical activities of proteins during denaturation at different pH's have given more detailed elucidation of the mechanism of denaturation.

The pH dependence of denaturation was explained by considering the ionization of PAS and protein. The effects of sulfur linkage of the protein molecule on the denaturation were discussed from the point of view that the rearrangement of the peptide chains of the protein molecule by denaturation was restricted by sulfur linkage.

Consequently, it was concluded that the partial unfolding of the peptide chains which was assumed to occur in the initial stage of the denaturation by the increase in the dielectric constant of the solvent by PAS, was followed by a refolding or rebinding through the bound PAS. In the rearrangement of the peptide chains, the binding of PAS to protein played the most important role, controlling the rate and the extent of denaturation.

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Alteration of yeast cell protein by denaturing agents

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In microbiology the treatments for inhibition of microbial growth have been carried out by using the denaturing agents such as acids, bases, organic solvents, salts of heavy metals, detergents, and other compounds as well as by heating, irradiation with ultraviolet rays or ultrasonic waves, and other physical means. It is commonly considered that the denaturing agents bring about the alteration of the microbial cell proteins and disturb the growth. By means of the determination of phosphorus or nitrogen flowing out of the cells, Hotchkiss[1] has demonstrated that certain detergents disrupted the surface structures of bacterial cells.

The author studied the effects of cations on the penetration of Na ion into the yeast cells and found that the cells took a larger amount of Na ion when certain heavy metal ions were present than when Ba, Ca, or Mg ion was present or when no other ions were

Table 1. Amounts of Na ion taken in yeast cell in the presence of various cations.

Metallic salt	Conc. of metallic salt	Na ion in cell*
$BaCl_2$	1×10^{-1}M	2.0 mg.
$CaCl_2$	1×10^{-1}	2.0
$MgCl_2$	1×10^{-1}	2.0
$CuCl_2$	6×10^{-2}	3.0
$HgCl_2$	2×10^{-3}	3.6
$AgNO_3$	2×10^{-3}	3.5
Control	0	2.0

* Amount of Na ion in cell is expressed as mg. of NaCl/100 mg. of yeast cell.

(Table 1). It was considered that this phenomenon resulted from the alteration of the yeast cells by heavy metals which act as denaturing agents of proteins. Therefore the changes of the amount of Na ion taken in cells, brought about not only by heavy metals but also by some other kinds of denaturing agents, were studied.

In microbiology it is a well-known process to distinguish living cells from dead cells by using certain dyes. After denaturation some proteins change their original capacity to combine with certain dyes.[2~5] Oster[6] has reported that fluoresceins (eosine, erythrosine and Bengal rose) hardly combined with intact tobacco mosaic virus or egg white, but that they combined with heated proteins and moreover modified their absorption spectra by the combination.

In this paper, therefore, the relationship between the adsorption of eosine yellowish by yeast cells and the denaturation of the cell proteins was also studied. In addition to the above experiments, the change of the activity of SH groups contained in yeast cells was studied, because the denaturation of protein is frequently accompanied by the increase of the activity of the groups.

Experimental method

1) Amount of Na ion taken in yeast cell

After centrifuging the cultures of *Saccharomyces sake* (Peptone-Hayduck medium,* 30°C., 40 hrs.), the cells were washed with distilled water 3 times and suspended in distilled water. Five ml. of cell suspension (including about 100 mg. wet cells), after the denaturing agent being added to it, was left for 5 min. at room temperature (15~16°C.). After centrifuging, the cells were washed with distilled water 2 times, and suspended again in 10 ml. of distilled water. Then 10 ml. of 2 M NaCl solution was added to the suspension, and the latter was left for 10 min. The suspension was filtered with No. 4 glass filter by using a strong sucker. The external moisture was removed from the yeast cells during the process of the filtration (the layer of the cells on the glass filter was less than 1 mm. thick). Fifty mg. of the treated cells was suspended in 20 ml. of distilled water, the Na ion content in this suspension was estimated by means of flame photometry at 589 mμ, and the amount of Na ion taken by yeast cells was calculated. In this case the cellular components scarcely interfere with this flame photometry (Table 2). The effect of heating was studied with the cell suspension which was heated in water bath at test temperature for 5 min., then cooled quickly down to room temperature.

* Peptone, 1%; $MgSO_4 \cdot 7H_2O$, 0.3%; KH_2PO_4, 0.1%; Glucose, 5%

Table 2. Effect of yeast cell components on the flame photometry of Na ion.

No.	Component of sample	Conc. of Na ion
1	10 ml. of 3.4×10^{-3}M NaCl + 50 mg. washed cell	101.8%
2	〃 +50 mg. cell heated to 90°C.	97.5
3	〃 +50 mg. cell exposed to M/10 KH_2PO_4*	102.0
4	〃 +50 mg. cell exposed to 2 M H_2SO_4*	102.2
5	〃 +50 mg. cell exposed to 3×10^{-2}M $CaCl_2$*	103.3
6	〃 +50 mg. cell exposed to 2×10^{-3}M $HgCl_2$	102.0
7	〃 +50 mg. cell exposed to 50% Ethanol	104.5
8	10 ml. of 3.4×10^{-3}M NaCl (Control)	100.0

* It has been reported that a large amount of P, Ca or strong acid interferes with the Na determination by flame photometric method.

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When the yeast cells were suspended in 1 M NaCl solution, the amount of Na ion taken in cells was 2 mg. expressed as NaCl per 100 mg. of the cells, and the percentage of the moisture of the cells was 46, therefore the concentration of the Na ion in cell was about 75 per cent of that of the external environment.

2) *Adsorption of eosine by cells*

The denaturing agent was added to 5 ml. of washed cell suspension, and the suspension was left for 10 min. The cells were centrifuged, washed with M/50 phosphate buffer solution (pH 5.4) 2 times, and suspended in 10 ml. of the same phosphate buffer solution. Ten ml. of 2×10^{-5}M eosine yellowish solution was added to this suspension, and the optical density of the centrifuged supernatant of the suspension was determined at 514 mμ after 20 min.

3) *Activity of SH groups*

After the addition of the denaturing agent, 10 ml. of the cell suspension (dry weight 63.6 mg.) was left for 10 min., then the cells were washed with distilled water 2 times and suspended in 18 ml. of distilled water. One ml. of 8 M ammoniac solution and 1 ml. of 2 M NH_4No_3 solution were added to this suspension, and the SH groups contained in the cells were determined by means of amperometric titration with 10^{-3} M $AgNO_3$ solution.

4) *Fungicidal action of denaturing agents*

Yeast cells (dry weight 10 mg.) were suspended in 5 ml. of distilled water containing various amounts of denaturing agents, and left for 10 min., then 2 drops of the suspension were inoculated to 5 ml. of fresh Peptone-Hayduck medium, and the turbidity of the medium was inspected after 2 days at 30°C. In the case of acid or alkaline treatment, the cell suspensions were neutralized with dilute NaOH or H_2SO_4 solution and then 2 drops of the suspension were inoculated. In the heating test, the cell suspension was heated in water bath at test temperature for 5 min., and cooled quickly down to room temperature and then 2 drops were inoculated. The fungicidal activity of $HgCl_2$ was also determined after one of the following treatments being carried out.

(1) Cysteine-HCl (0~50 mg. in weight) was added to the cell suspension exposed to $HgCl_2$ and then 2 drops of the suspension were inoculated to 5 ml. of fresh medium.

(2) The cells exposed to $HgCl_2$ were washed 3 times with water and the cells were suspended in 5 ml. of distilled water and then 2 drops were inoculated.

(3) Two drops of the cell suspension exposed to $HgCl_2$ were inoculated to 5 ml. of fresh medium containing 0~5 mg. of cysteine-HCl. The results showed that hardly any difference could be found in the fungicidal activity of $HgCl_2$, whether treated with cysteine or with water or without either.

Results and discussion

In these experiments the change of the amount of Na ion taken in cell has been applied to the indication of the denaturation of yeast cell proteins, which was formerly done by the increase of SH groups or the change of adsorption of eosine. The denaturation by $HgCl_2$ or formaldehyde was indicated by the change of the amount of Na ion in cell and of the adsorption of the dye, because the chemicals react to the SH groups contained in the undenatured and denatured yeast cell proteins. The mechanism by which the amount of Na ion taken in yeast cell is changed remarkably when the cells are exposed to certain chemical or physical treatments is not yet certainly known.

As a result of the comparison between the concentration of denaturing agent to show the fungicidal action and the concentration at which the denaturation of yeast cell proteins was brought about, it was found that the chemicals fell in 2 types. One typical agent was alcohol, the action of which on the yeast cells was similar to that of heating. Alcohol, even below the fungicidal concentration, brought about the denaturation of cell proteins, and the concentration of alcohol

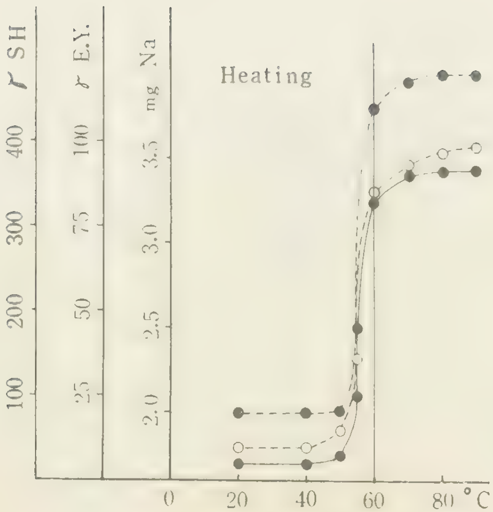


Fig. 1

Figs. 1～8. Effects of denaturing agents and heating on the amount of Na ion in yeast cell, adsorption of eosine by cell and activity of SH groups of cell protein.

●——● SH group (expressed as γ of cysteine) liberated from dry weight 63.6 mg. of yeast cells.

○----○ Eosine Yellowish adsorbed by yeast cell
Dry weight of yeast cell used for

Heating	54.3 mg.	Mercuric chloride	54.3 mg.
Ethanol	„	Formaldehyde	„
Acetone	„	Sulfuric acid	1.8
Phenol	„	Potassium hydroxide	„

●----● Na ion (expressed as mg. of NaCl/100 mg. of yeast cells) taken in yeast cell

The longitudinal line in each figure (Heating, 60°C.; Ethanol, 35%; Acetone, 35%; Phenol, 0.2 M; Sulfuric acid, 0.9 M; Potassium hydroxide, 0.26 M; Mercuric chloride, 0.001 M; Formaldehyde 0.5 M) shows the minimum temperature or concentration to show the fungicidal action.

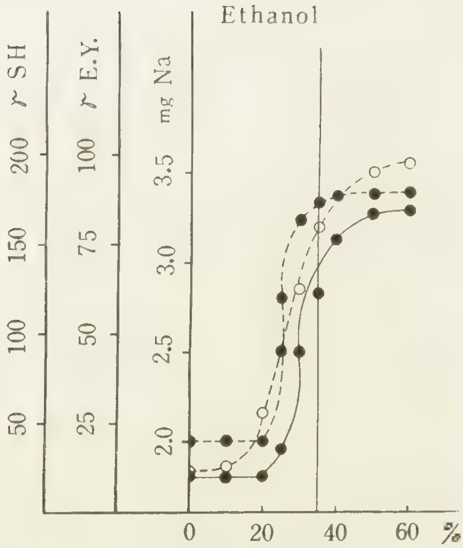

Ethanol
γ SH
γ E.Y.
mg Na
50 100 150 200
25 50 75 100
2.0 2.5 3.0 3.5
0 20 40 60 %

Fig. 2

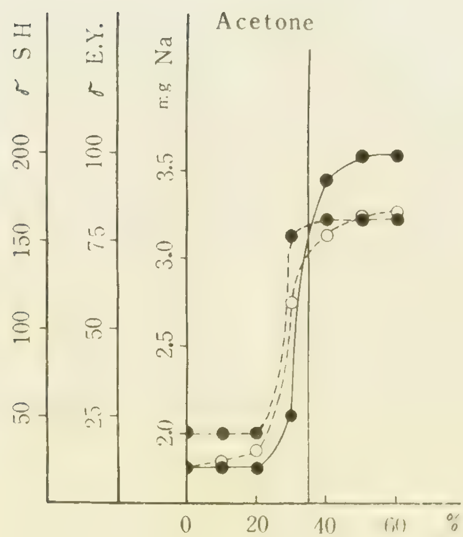

Acetone
γ SH
γ E.Y.
mg Na
50 100 150 200
25 50 75 100
2.0 2.5 3.0 3.5
0 20 40 60 %

Fig. 3

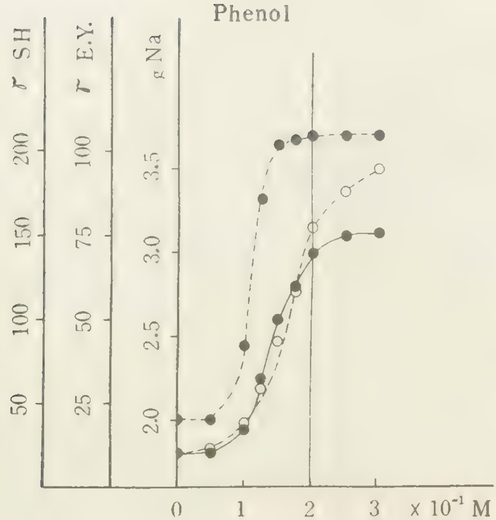

Phenol
γ SH
γ E.Y.
g Na
50
100
150
200
25
50
75
100
2.0
2.5
3.0
3.5
0
1
2
3
x 10⁻¹ M

Fig. 4

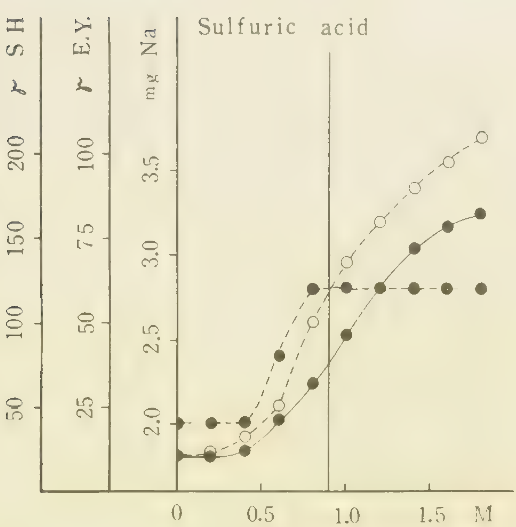
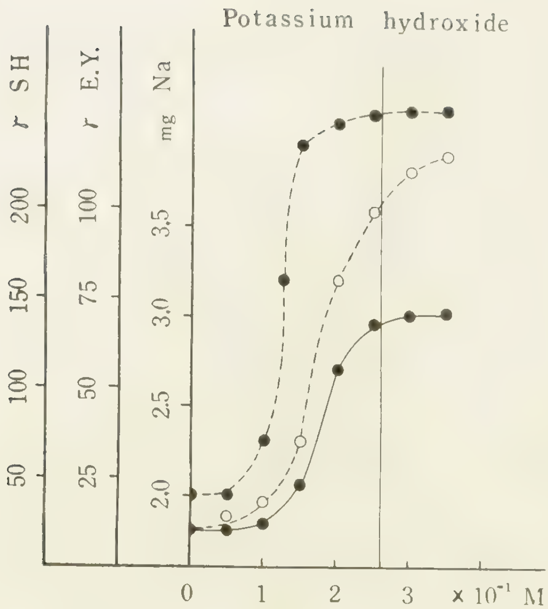
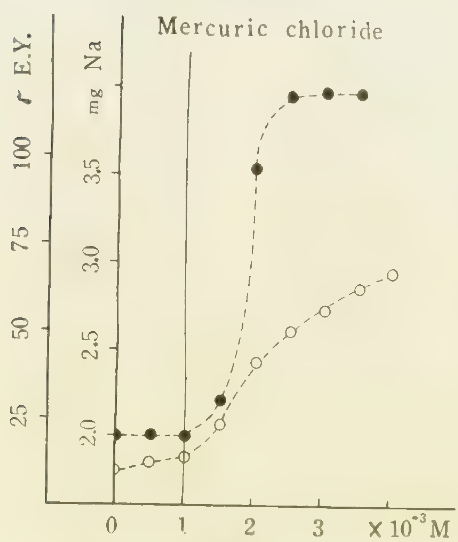

Sulfuric acid
γ SH
γ E.Y.
mg Na
50
100
150
200
25
50
75
100
2.0
2.5
3.0
3.5
0
0.5
1.0
1.5
M

Fig. 5



Potassium hydroxide
r SH
r E.Y.
mg Na
200
150
100
50
100
75
50
25
3.5
3.0
2.5
2.0
0
1
2
3
× 10⁻¹ M

Fig. 6



Mercuric chloride
r E.Y.
mg Na
100
75
50
25
3.5
3.0
2.5
2.0
0
1
2
3
× 10⁻³ M

Fig. 7

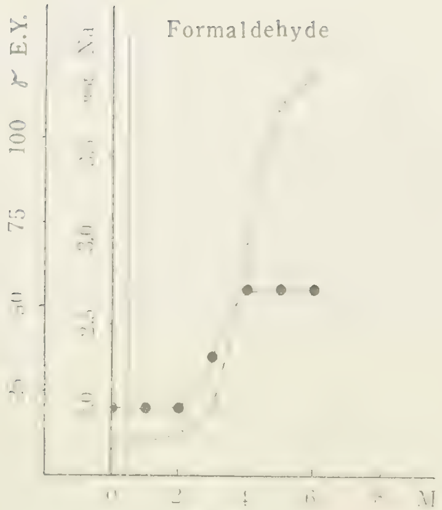


Fig. 8

in which the complete denaturation of the cell proteins arose was almost the same as the fungicidal concentration. Acetone, acid, base and phenol fell within this type. The fungicidal actions of these agents and of heating were due probably to their alterative influence exercised on the fungal cell proteins. There are some theories which contribute to the explanation of the fungicidal action of heating in the wet condition, such as protein denaturation theory, poisoning theory, asphyxiation theory, lipid liberation theory. The author's experimental results may help to give a foundation to the denaturation theory.

$HgCl_2$ and formaldehyde fell in the other type, and showed strong fungicidal action even at the concentration below that which is effective in causing the denaturation of cell proteins. It has been reported that the bacterial cells which have apparently lost their reproductive capability by Hg ion regain it when the cells have been washed with water or exposed to such sulfhydryl compounds as cysteine or glutathione.[7] It was confirmed by my experiments that, when the $HgCl_2$ treated cells were exposed to the sulfhydryl compounds, the concentration of $HgCl_2$ to show the fungicidal action was also below that which is effective in causing the denaturation of cell proteins. Therefore, it can be reasonably considered that the reaction of Hg ion to cell proteins is irreversible even at the concentration at which the denaturation of the proteins never arises.

Summary

1. The fungicidal action of 7 sorts of denaturing agents and heat treatment were studied concerning their capacity of alternating fungal cell proteins.

2. The alteration of yeast cell proteins was indicated by the change of the amount of Na ion taken in cells.

3. The comparison between the concentration to show the fungicidal action and the concentration at which the denaturation of the cell proteins arose showed that the denaturing chemicals fell in 2 types.

Acknowledgment

This investigation was completed at the Chemical Laboratory of Aquatic Products, Faculty of Agriculture, Kyushu University, under the direction of Professor Yukio Tomiyasu. The author wishes to express sincere gratitude for his kind guidance during the course of the investigation.

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Studies on the flying-fishes of the Amakusa Islands. Part 1. Faunal discussion with the life-historical notes[1, 2]

Hiroshi Tsukahara

The present paper is a descriptive list of the flying-fishes collected off the western coast of the Amakusa Islands, Kyushu Province, Japan. Several species of the flying-fish have considerable importance economically to the fishery in this region. This fishery operates mainlys by the gill net during the summer. During my recent studies on the economical fishes of the Amakusa Islands, I began to be interested in the habits and the life histories of flying-fishes and attempted to survey the flying-fish fauna in this region. Imai (1955) recorded nineteen species in the larval stage off the southern waters of Kyushu Province, but in Amakusa region a knowledge about the flying-fish fauna was not sufficient. It will be a pleasure to me if the present study can add something to the knowledge of geographical distribution and the life histories of these flying-fishes.

This report includes thirteen species; five adult forms of the genera *Parexocoetus*, *Cypselurus* and *Prognichthys*, and thirteen immature forms of the genera *Oxyporhamphus*, *Parexocoetus*, *Exocoetus*, *Cypselurus*, *Prognichthys* and *Hirundichthys* collected by myself in 1955.

The adult form was taken by the gill net and the young, juvenile and larval forms were captured mainly by a dip net with the aid of a flood light at night. I found these methods were useful especially for collecting the flying-fishes in the various stages. Besides, collection of the juvenile was made by a dip net from their favorite hiding

[1] Contribution from the Fisheries Laboratory, Faculty of Agriculture, Kyushu University.

[2] Contribution from the Amakusa Marine Biological Laboratory, Faculty of Science, Kyushu University. No. 99.

places in the floating sea-weeds. The work has been carried out in the Amakusa Marine Biological Laboratory, Faculty of Science, Kyushu University. The Amakusa Islands are situated in the warmer temperate region, and the western coast of the outer main island opens to the East China Sea. The southern tropical fishes are brought up by Tsushima Warm Current, so that these fish fauna are rich.

Here, I wish to express my sincere thanks to Prof. Keitaro Uchida and Prof. Hiroaki Aikawa, Laboratory of Fisheries, Kyushu University, for their guidance. I also extend my thanks to Assist. Prof. Sadahiko Imai, Faculty of Fisheries, Kagoshima University for many valuable suggestions, and to Assist. Tsukasa Shiokawa and the other staff of the Amakusa Marine Biological Laboratory for their assistance in collections and technical procedures.

The list of species described is as follows;

	Scientific Name	Common Name
1.	*Oxyporhamphus micropterus micropterus* (Cuvier et Valenciennes)	Sayori-tobiuo
2.	*Parexocoetus mento mento* (Valenciennes)	Bashō-tobiuo
3.	*Parexocoetus brachypterus brachypterus* (Richardson)	Tsumari-tobiuo
4.	*Exocoetus monocirrhus* (Richardson)	Hagoromo-tobiuo
5.	*Cypselurus heterurus döderleini* (Steindacher)	Tsukushi-tobiuo
6.	*Cypselurus katoptron* (Bleeker)	Sannoji-damashi
7.	*Cypselurus opisthopus hiraii* Abe	Hoso-tobi
8.	*Cypselurus naresii* (Günther)	Uchida-tobiuo
9.	*Cypselurus starksi* Abe	Ariake-tobiuo
10.	*Cypselurus poecilopterus* (Cuvier et Valenciennes)	Aya-tobiuo
11.	*Prognichthys agoo* (Temminck et Schlegel)	Tobiuo
12.	*Prognichthys seali* Abe	Daruma-tobiuo
13.	*Hirundichthys oxycephalus* (Bleeker)	Hosoao-tobi

Oxyporhamphus micropterus micropterus (Cuvier et Valenciennes)

The specimens from this district are all immature forms and I have nine specimens, ranging from 50 mm. up to 80 mm. in fork length. These specimens were taken in July and August.

The body of the juvenile is elongated, compressed and the lower jaw produced as those in the halfbeaks, *Hemiramphidae*, and the lower lobe of caudal fin is more prolonged than the upper. The pectoral and ventral fins are short (Figs. 1, 2).

The ray counts are: Dorsal 14, Anal 14, Pectoral 12, Ventral 6.

The coloration of the body in life is bright silvery and the part of trunk is bright bluish. They were attracted to a flood light at

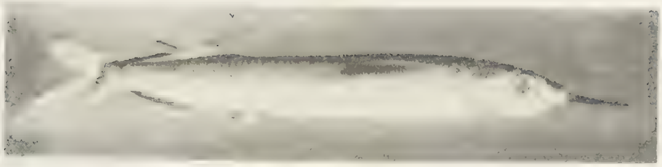

Fig. 1. Juvenile of *Oxyporhamphus micropterus micropterus.* ×1

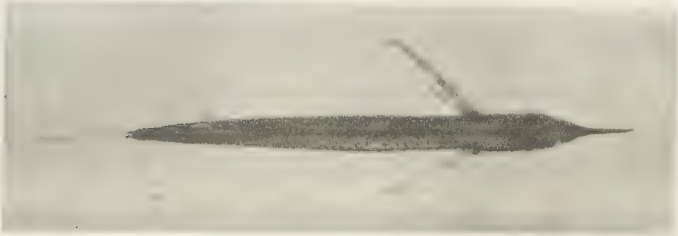

Fig. 2. Dorsal view of juvenile. ×1

night and captured by a dip net when they lay motionless close to the water surface. They occasionally made short, sporadic flights, similar to that of *Hemirhamphidae.*

Parexocoetus mento mento (Valenciennes)

Maturing adults were collected in the period from May to October and the spawned-out were observed in this region and my material shows the series of developmental stages. This species is the smallest in size in adult among the flying-fishes taken from this region, the largest being 130 mm. in the female and 123 mm. in the male in fork length, so far as I have examined. They feed entirely on planktonic organisms, mostly copepods.

The shape of the body in adult stage is rather long and compressed; Fig. 3 shows the general appearance of the species. The dorsal fin is remarkably high and the pectoral fins are oblong and of moderate length. The ventral fins are rather short and situated somewhat far forward on the body.

Fig. 3. Adult of *Parexocoetus mento mento.* Fork length 12 cm.

The ray counts are: Dorsal 9—10, Anal 11—12, Pectoral 12, Ventral 6.

The coloration of the body in life is dark bluish above and silvery white below and all faint yellowish. The dorsal fin is quite black excluding the base. The upper part of the pectoral is faint grayish and the ventral fins are hyaline.

The spawning season occurs from May to September and the peak is in June and July. The eggs were stripped and inseminated with the milt from the male. The embryonic development was observed and the hatched larvae were reared in the aquarium. The egg is spherical, 1.75—1.78 mm. in diameters, with about 20 long entangling tendrils distributed over the whole surface (Fig. 4). The yolk is transparent and the oil-globule absent.

Fig. 4. Eggs of *Parexocoetus mento mento.* ×ca. 10

The number of eggs in the ovary is presumed to be from about 1,000 to 2,000. The spawning seems to take place at once from the sunset to the next morning. It requires about 10 days at 21—25 C. water temperature to hatch out. The hatching-out takes place in several hours after the sunset. The newly hatched larvae were 4.5—5.2 mm. in total length. In the larval stages the body is a little more robust and the fin rays have definitely developed, except the pectoral fins. The coloration of the body is dark brownish above and bright yellow brownish below. The ventral fins are dark greenish. Immature forms

from 10 mm. up to 48 mm. in fork length were captured numerously by a dip net under a night light. The juvenile appeared from early July to late October (Fig. 5).

Fig. 5. Eggs, larvae and juveniles of *Parexocoetus mento mento.* ×ca. 2

Fig. 6. Dorsal view of prolonged lower jaw. ×20

In the juvenile stages, the elevation of dorsal fin increases and the dorsal fin is wholly black, except near the base. The ventral fins are large and almost brownish. The characteristics of pigmentation are the three rows of melanophores which are situated along the dorsal, anal bases and the lateral portion of posterior body. The barbel of the lower jaw is absent, but the lower jaw produced a little as in the saury, *Scombresocidae* (Fig. 6). These small fishes spread out the four "wings" and could leap into the air for a distance of a foot. In the specimens longer than 25 mm. in fork length, the shape of body agrees with the adult form and is dark bluish above and silvery white below (Figs. 7, 8).

Fig. 7. Young of *Parexocoetus mento mento.* ×2

The growth is so rapid that the fishes almost become matured in one year. They spawned at the end of their first year and most of them seem to die after spawning. Maturity is reached at the size of about 98 mm. in fork length in the male and 119 mm. in the female.

The young shows a tendency to leave this region in winter migrating towards the southern waters.

Fig. 8. Dorsal view of young. ×2

Parexocoetus brachypterus brachypterus (Richardson)

The specimens are all small in size, and ranges from 25 to 60 mm. in fork length are taken in July. Imai (1955) has recorded the capture of adults off the southern region of Kyushu, but I could not collect the adults in Amakusa region.

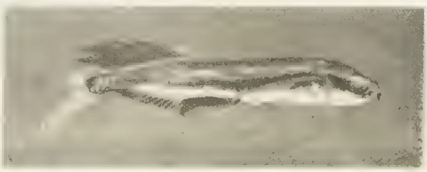

Fig. 9. Juvenile of *Parexocoetus brachypterus brachypterus.* ×1

The body of a juvenile is rather long and compressed. The mid-most ray of the dorsal fin is greatly prolonged, thus giving a remarkably high shape to the fin. The pectoral fins are rather short. The ventral fins are relatively long, although those of the adult rather short (Figs. 9, 10). During earlier stages, the lower jaw has a pair of short, black barbel (Fig. 11).

Fig. 10. Dorsal view of juvenile. ×1

Fig. 11. Dorsal view of barbel of lower jaw. ×10

The ray counts are: Dorsal 13, Anal 13, Pectoral 12, Ventral 6.

The coloration of the body is dark above and silvery white below and a dark band of pigment across the eye extends to the middle part of the caudal peduncle. The dorsal fin is quite black and the anal fin is black, except in the proximal part, which is hyaline. The pectoral and ventral fins are grayish.

Two juvenile forms of *Parexocoetus*, *P. brachypterus brachypterus* and *P. mento mento*, were found in this region and in the same season. During the juvenile stage, this species is darker in the color of body and higher in dorsal fin than *P. mento mento* and has a pair of barbel under the lower jaw, but the latter is absent.

Exocoetus monocirrhus (Richardson)

The present material is all juvenile, with a maximum fork length of 58 mm. which was taken in July and August, and adults were not collected. They were captured by a dip net when they lay motionless under a night light with the pectoral fins spread out and the barbel of lower jaw prolonged anteriorly.

The body of a juvenile is short and high, and the head is elevated steeply behind the eyes. The pectoral fins are very long and the ventral fins are short and anteriorly situated (Figs. 12, 13). An

Fig. 12. Juvenile of *Exocoetus monocirrhus*. ×1

Fig. 13. Dorsal view of juvenile. ×1

unpaired barbel of the lower jaw is rather long and slightly expanded laterally with the tip being roundish and quite black (Fig. 14).

Fig. 14. Dorsal view of barbel of lower jaw. ×5

The ray counts are: Dorsal 13, Anal 13, Pectoral 15, Ventral 6.

The coloration of the body in life is brownish blue above and silvery white below, with three obscure vertical black bars on the sides. The pectoral fins are faint grayish with a black vertical band on the middle part, a black line runs along the posterior margin with black parts on the base (Fig. 12). The ventral fins are hyaline. The dorsal fin is nearly transparent, with an obscure black bar. Sometimes these small fishes would glide in the air for a distance of at least 10 feet before striking the water again under a night light.

Cypselurus heterurus döderleini (Steindachner)

This is the larger form and is of economical importance among the flying-fishes in this region. I have a large series of developmental stages.

The body of the adult is rather slender and the belly is angular along the ventral edge; Fig. 15 shows the general appearance of the species. The pectoral fins are long and reach past rear of dorsal base. The tip of the ventral fins reaches the base of the 9th anal fin-ray. The origin of the anal fin is far behind that of the dorsal fin.

Fig. 15. Adult of *Cypselurus heterurus döderleini*. Fork length 28 cm.

The ray counts are: Dorsal 12–14, Anal 9–11, Pectoral 15–17, Ventral 6. The number of vertebrae (including the urostyle) is 47–49.

The coloration of the body in life is dark bluish above and silvery white below. The pectoral fins are faint greyish violet with the exception of the central part, margin and lower part being lighter. The dorsal, anal and ventral fins are greyish. They feed entirely on planktonic organisms, mostly copepods. The adult in this region is in spawning condition and the spawning takes place in May, June and July. The number of eggs carried by an adult female varied from about 8,500 to 10,000. The eggs were stripped and inseminated with milt from the male. The embryonic development was observed and the hatched larvae were reared in the aquarium, fed with the nauplii of the brine shrimp.

The eggs are spherical, on the average 1.86 mm. in diameter, having about 50 long tendrils attached uniformly over the whole surface (Fig. 16). The hatching-out takes place in about 14 days at 20° 22°C. water temperature and in several hours after the sunset. The newly hatched larvae were about 6.3 mm. in total length and swam in the water surface and darted about very actively in the aquarium. In the

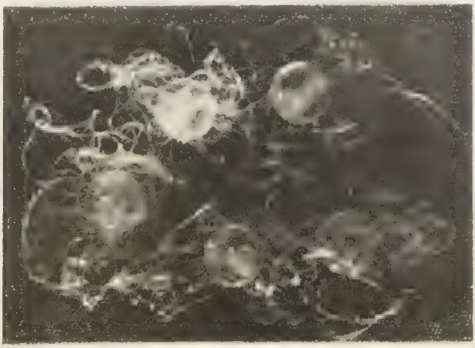

Fig. 16. Eggs of *Cypselurus heterurus doderleini*. ×ca. 5

larval stages, the shape of the body is a little robust, and with the most striking variation in color pattern, as all white, cream yellow, orange yellow, brown and dark brown. The pectoral and ventral fins are relatively large and the color is red brownish. In 25 days it attains about 14 mm. in total length and the caudal fin had begun to differenciate, lower lobe becoming longer. At this stage any disturbance in the water of the aquarium would cause these small fish to jump out of the water and glide for a short distance in the air.

Immature forms, from 14 to 110 mm. in fork length were captured numerously by a dip net under a night light. They swam sluggishly about near the light, so that they were very easily captured by a dip

Fig. 17. Eggs, larvae and juveniles of *Cypselurus heterurus dölerleini.* × ca. 2

net. In the juvenile stages, the body is slender and the coloration is bright orange yellow and has five vertical black bands on the sides.

These bands are continuous with the black parts of the dorsal and anal fins (Figs. 17, 18, 19). The juvenile larger than 15 mm. in fork length has a yellow paired barbel with the outer black flap, which is retained in the young fish, 110 mm. in fork length (Fig. 20).

In the young fish longer than 150 mm. in fork length, the shape and the color of the body nearly agree with the adult, although the middle part of the pectoral fin is pale (Fig. 21). These young fishes are of great importance in the western waters of Kyushu during October and November, and they migrate to the southern waters in winter.

Fig. 18. Juvenile of *Cypselurus heterurus dölerleini.* × 1

Fig. 19. Dorsal view of juvenile. × 1

Growth is very rapid and in one month it attains about 20 mm., in three months about 80 mm. and in one full year 250–300 mm. in fork length. Most fishes become matured and spawn at the end of the first year.

Fig. 20. Dorsal view of barbel of lower jaw. ×5

Fig. 21. Dorsal view of young. Fork length 18 cm.

Cypselurus katoptron (Bleeker)

Those at hand are 6 specimens ranging from 35 to 110 mm. in fork length, but the adult was not collected. These specimens were

Fig. 22. Juvenile of *Cypselurus katoptron*. ×1.5

Fig. 23. Dorsal view of juvenile. ×1.5

taken in July. They were attracted to a night light with the pectoral and ventral fins spread out and a paired barbel was held forward and slightly downward. The body of the juvenile is rather robust and the pectoral and ventral fins are large (Figs. 22, 23).

The ray counts are: Dorsal 14, Anal 10, Pectoral 15, Ventral 6.

In the juvenile, the coloration of the body in life is silvery yellow, with three obscure vertical black bands on the sides. These three

Fig. 24. Dorsal view of barbel of lower jaw. ×5

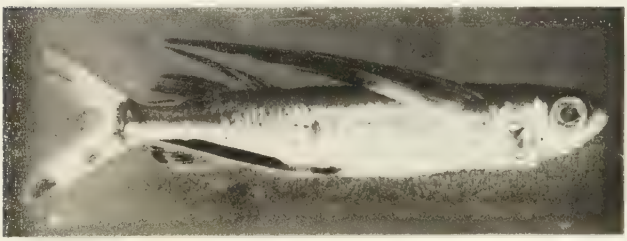

Fig. 25. Young of *Cypselurus katoptron*. ×1

Fig. 26. Dorsal view of young. ×1

bands are continuous with the black parts of the dorsal and anal fins. The pectoral fins have a yellow part at its midmost part. During

earlier stages the lower jaw has a pair of triangular yellow barbel with the margin black (Fig. 24). In the stages longer than 75 mm. in fork length, the shape of body is slender and the coloration is black bluish above and silvery white below with the barbel being lost (Figs. 25, 26).

Cypselurus opisthopus hiraii Abe

This species is the most important economically among the flyingfishes in this region and I have a large series of material indicating the life history. The adult form is medium sized among the flyingfishes and the body is rather slender; Fig. 27 shows the general appearance of the species. The pectoral fins are long and the tip reaches at the middle of dorsal base.

Fig. 27. Adult of *Cypselurus opisthopus hiraii*. Fork length 22 cm.

The ray counts are: Dorsal 11—14, Anal 9—10, Pectoral 14—15, Ventral 6. The number of vertebrae (including urostyle) is 45—46.

The coloration of the body is dark bluish above and silvery white below. The pectoral fins are greyish violet, except the lower part is transparent. The dorsal, anal and ventral fins are greyish. The adults that appeared in this region are in spawning condition and the spawning takes place in May, June and July. The number of the eggs carried out by an adult female varied from about 4,000 to 7,500.

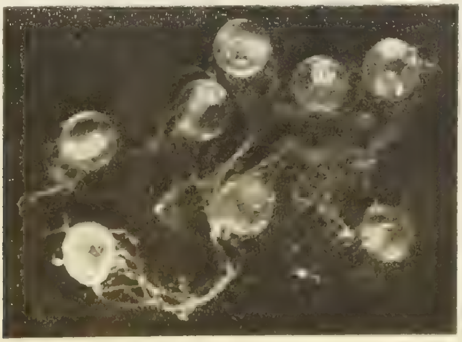

Fig. 28. Eggs of *Cypselurus opisthopus hiraii*. ×ca. 5

The eggs are spherical on average 1.47 mm. in diameter and have about 50 long tendrils attached uniformly over the whole surface (Fig. 28).

The eggs were stripped and inseminated with the milt from the male and the development was observed and the hatched larvae were reared in the aquarium, fed with the nauplii of the brine shrimp. The hatch-out takes place in about 14 days at 20°—22°C. water temperature and in several hours after the sunset. The newly hatched larvae were about 4.7 mm. in total length.

Abe (1955) considered that the natural hybrids between the present species and *Cypselurus heterurus döderleini* seem to occur infrequently, from the two points that the spawning season of both species agrees and intermediate forms sometimes occur in nature. I experimented on the hybrids between the present species and *C. heterurus döderleini* or *C. starksi*. The crossing was easily done on the mature egg and sperm, and the embryonic development was apparently normal, but the all hatched larvae died in the postlarval stage. I have not seen the intermediate form in nature during my studies.

Fig. 29. Eggs, larvae and juveniles of *Cypselurus opisthopus hiraii*. ca. 2

In the larval stage, the body is rather slender and reveals the temporary variation of color from nearly white to dark brown. The pectoral and ventral fins are relatively large and brownish. They swam close to the surface of water with the pectoral and ventral fins widely spread and darted about very actively by the rapid movement of the caudal peduncle (Fig. 29). In 3 days, attaining about 7 mm. in total length, they show a tendency to attach to the sea-weeds (Fig. 30). By the sudden disturbance in water, one-month old larva, about 20 mm. in fork length, leaped out beyond the fence of the aquarium of which 15 cm. high above the water.

The juvenile forms, from 20 to 105 mm. in fork length were captured numerously by a dip net under a night light. In the juvenile stages, the body is slender and the coloration is dark brownish above and black below. The juvenile longer than 12 mm. in fork length, it has a black rounded barbel (Fig. 31).

Fig. 30. Larvae attached to the sea-weed in aquarium. ×1

In advanced stages, the coloration of the body is dark bluish above, silvery on side with a longitudinal black band, and black below. The pectoral fins are black, except the lower transparent part. The ventral fins are black (Figs. 32, 33). The young, longer than 100 mm. in fork length, lacks the barbel on the lower jaw and the shape of the body and the coloration agree with the adult form, dark bluish above, silvery white below.

The growth is very rapid and in a

Fig. 32. Juvenile of *Cypselurus opisthopus hiraii*. ×1

Fig. 31. Dorsal view of barbel of lower jaw. ×12

Fig. 33. Dorsal view of juvenile. ×1

month it attains about 20 mm., in three months 80 mm. and in one full year 200 240 mm. in fork length and the most of them seem to mature and spawn. These four or five month old fishes are important economically in the western waters of Kyushu during October and November, and then they show a tendency to leave there for the southern waters in winter.

Cypselurus naresii (Günther)

The present materials are all juvenile with a maximum fork length of 73 mm. and were taken in July, and the adult was not collected. The general appearance in juvenile is similar to the juvenile of *C. opisthopus hiraii*, but is distinguished by the number of vertebrae and the barbel of lower jaw. The body of the juvenile is rather slender (Figs. 34, 35). The pectoral fins are large and the tip reached to the base of lower rudimentary caudal fin-ray.

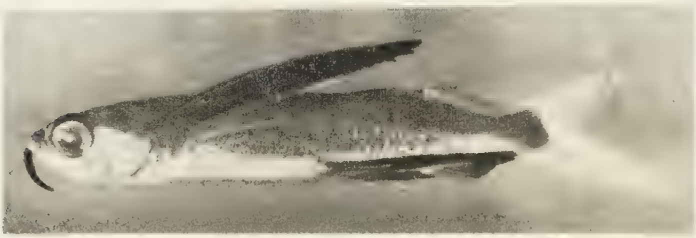

Fig. 34. Juvenile of *Cypselurus naresii.* ×1.2

Fig. 35. Dorsal view of juvenile. ×1

The ray counts are: Dorsal 12, Anal 10, Pectoral 15, Ventral 6. The number of vertebrae (including urostyle) is 42.

The coloration of the body is dark bluish above, silvery white below and has a longitudinal black band on the side. The pectoral fins are black except the lower transparent part. The ventral fins are black. The lower jaw has a considerable long black barbel like a ribbon

(Fig. 36). They were attracted to a light at night with the pectoral and ventral fins spread out and the barbel of lower jaw protruded anteriorly.

Fig. 36. Dorsal view of barbel of lower jaw. ×5

Cypselurus starksi Abe

The present species is of economical importance among the flying-fishes in this region. I have a large series of materials indicating the life history.

The adult was captured from June to August. The adult form is rather robust and medium in size among the flying-fishes with the snout rather blunt; Fig. 37 shows the general appearance. The pectoral fins are long and broad, and the tip reaches the posterior end of the dorsal base.

Fig. 37. Adult of *Cypselurus starksi.* Fork length 20 cm.

The ray counts are: Dorsal 12 14, Anal 8 9, Pectoral 15 16, Ventral 6. The number of vertebrae (including urostyle) is 43 44.

The coloration of the body is dark bluish above and silvery white below. The pectoral fins are bluish black and the lower part is transparent. The dorsal, anal and ventral fins are greyish.

The spawning season commences in early July and ends in August, and the spawning act seems to take place at once after the sunset till the next morning. The number of the eggs carried by one female

varied from about 4,000 to 8,000. The eggs were stripped and inseminated with milt from the male and the embryonic development were observed. The hatched larvae were reared in the aquarium, fed with the nauplii of brine shrimp.

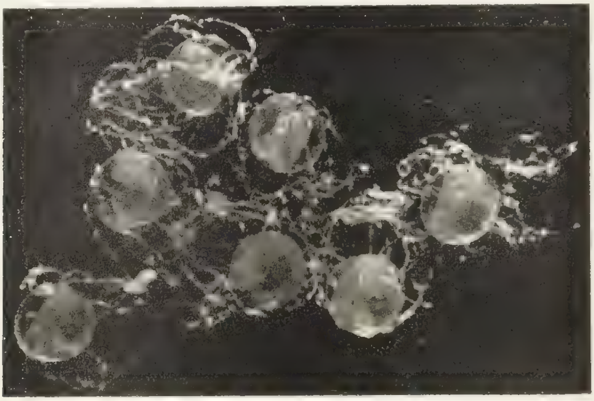

Fig. 38. Eggs of *Cypselurus starksi.* ×ca. 7

The egg is spherical, on average 1.66 mm. in diameter with about 50 long tendrils scattered over the whole surface (Fig. 38). The hatch-out takes place in about 6 days at 26° 28 C. water temperature and in several hours after the sunset.

Fig. 39. Eggs, larvae and juveniles of *Cypselurus starksi.* ×ca. 2

In the larval stages the body is robust and the coloration varies considerably among the reared specimens and shows temporary change from yellow to dark red-brown in color pattern. The pectoral fins are greyish with the basal part slightly brownish and the ventral fin is almost dark except the marginal part. The prolarva is very heavily pigmented (Fig. 39). In one month, it attained about 17 mm. in fork length and began to jump out of the water in the aquarium by a sudden disturbance.

Immature forms, from 23 98 mm. in fork length, were captured numerously by a dip net under a night light. In the juvenile stages,

the body is robust and in coloration the majority of them appear to be rather profusely dotted with black, although they are quite brownish. The fins are all well developed and the pectoral and ventral fins are dark bluish and the lower lobe of the caudal fin is elongated (Figs. 40,

Fig. 40. Juvenile of *Cypselurus starksi*. ×1

41). These juveniles lack the barbel of the lower jaw. In the young fish longer than 150 mm. in fork length, the body is very robust and the color agrees with the adult, but the ventral fins are black.

Fig. 41. Dorsal view of juvenile. ×1

The advanced young, 120–150 mm. in fork length, are economically important together with *C. opisthopus hiraii* and *C. heterurus döderleini* in the western waters of Kyushu during October and November. These young fishes probably migrate to the southern waters in winter. The growth is very rapid and in a month attains 18 mm., in three months 80 mm., in one full year 180–220 mm. in fork length and the most fishes seem to mature and spawn.

Cypselurus poecilopterus (Cuvier et Valenciennes)

The present materials are two juvenile specimens being 60, 70 mm. in fork length, captured in August and September. The adult form

was not collected. The spawning ground seems to be far southern waters from this region.

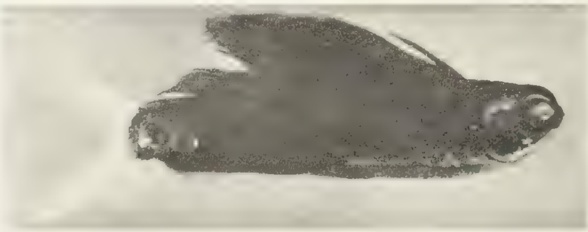

Fig. 42. Juvenile of *Cypselurus poecilopterus.* ×1

In the juvenile, the body is very robust and the belly is angular along the ventral edge and the caudal peduncle is high (Figs. 42, 43). The eyes are large. The tip of pectoral fin reaches a little beyond

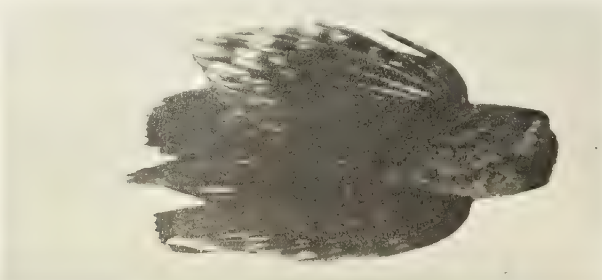

Fig. 43. Dorsal view of juvenile. ×1

the base of last dorsal fin-ray. The ventral fins are large and the tip reaches to the base of the anteriormost rudimentary fin-ray of the lower lobe of caudal fin.

The ray counts are: Dorsal 12, Anal 8, Pectoral 15, Ventral 6.

The coloration of the body in life is brownish black. The color of pectoral and ventral fins are dark brownish with brownish black spots. The dorsal and anal fins are dark brownish. The barbel of the lower jaw is absent.

Prognichthys agoo (Temminck et Schlegel)

This species is economically important in Japan, but not abundant in this region. The adult appeared in September and the juvenile was captured in July.

The adult form is rather slender and the largest in size in this region; Fig. 44 shows the general appearance. The pectoral fins are

long and the tip reaches a little beyond the base of dorsal fin. The tip of the ventral fin reaches the end of the last anal fin-ray. The origin of the anal fin is vertically below from the second to fourth of dorsal ray

Fig. 44. Adult of *Prognichthys agoo*. Fork length 33 cm

The ray counts are: Dorsal 10–12, Anal 11, Pectoral 17, Ventral 6. The number of vertebrae (including urostyle) is 46–48.

The coloration of the body is black bluish above and silvery white below. The pectoral fins are blue greyish and the lower part is lighter. The dorsal and ventral fins are grey and the anal fin is transparent.

In the juvenile, the shape of body is slender (Figs. 45, 46). The coloration is brownish blue above and silvery white below. Small

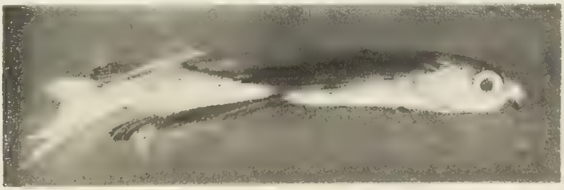

Fig. 45. Juvenile of *Prognichthys agoo*. ×1

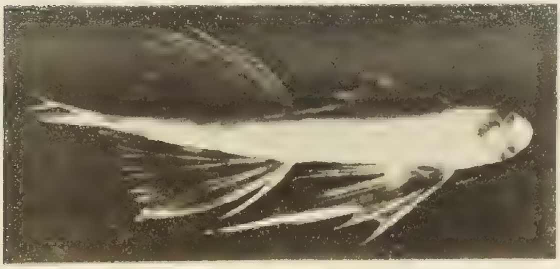

Fig. 46. Dorsal view of juvenile. ×1

melanophores are scattered over the body with five obscure black bars on the belly. The shape of pectoral fin is peculiar and is faint blackish with a quite black line along the margin and the upper part is lighter. The ventral fins are broad and the color is faint black, the

basal part paler. The lower jaw has a paired yellow barbel with black margin (Fig. 47).

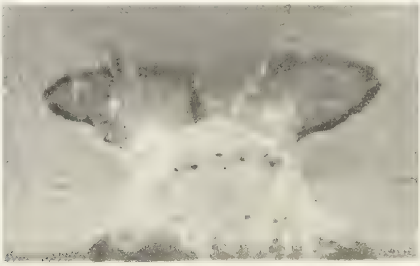

Fig. 47. Dorsal view of barbel of lower jaw. ×5

Prognichthys seali Abe

The present material is one juvenile specimen, 75 mm. in fork length, captured in July. Adult form was not collected. The body is very robust and the snout is short and the eyes are large (Figs. 48, 49). The posterior tip of pectoral fin reaches the base of last dorsal fin-ray. The tip of ventral fin reaches the origin of lower caudal fin-ray.

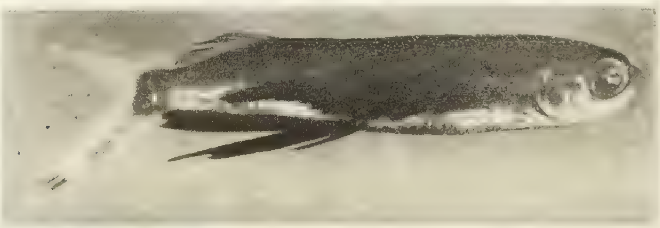

Fig. 48. Juvenile of *Prognichthys seali*. ×1

Fig. 49. Dorsal view of juvenile. ×1

The ray counts are: Dorsal 10, Anal 9, Pectoral 19, Ventral 6.

The coloration of the body is bluish black above and silvery below. The pectoral fin is black with the postero-dorsal margin hyaline. The ventral fin is all black. The barbel of the lower jaw is absent.

Hirundichthys oxycephalus (Bleeker)

The present material is of post-larvae, juveniles and young, ranging from 12 mm. up to 130 mm. in fork length. Immature forms are abound in this region in July, August and September, but the adult was not collected.

In the specimen measured 130 mm. in fork length, the shape of body is slender and similar to *Cypselurus opisthopus hiraii*, but differs from the latter in the position of the insertion of anal fin (Fig. 50). In the present species the anal fin is inserted nearly under the insertion of dorsal fin. The pectoral fins are long and reaches beyond the base of last dorsal finray. The tip of ventral fin reaches almost to the end of the anal base.

Fig. 50. Young of *Hirundichthys oxycephalus*. Fork length 13 cm.

The ray counts are: Dorsal 10–11, Anal 11–12, Pectoral 15–16, Ventral 6. The number of vertebrae (including urostyle) is 45–47.

The coloration of the body is dark bluish above and silvery white

Fig. 51 Dorsal view of larvae and juvenile of *Hirundichthys oxycephalus*. ×1

below. The pectoral fins are greyish purple, lower part transparent. The ventral fins are mostly transparent, the dorsal side being dark. The dorsal and anal fins are mostly transparent.

In the larval stages, the body is slender and the color is white with the melanophores scattered over the body. The pectoral and ventral fins are roundish with the margin black (Fig. 51). In the advanced form, the appearance of pectoral and ventral fins are shown in Figs. 52, 53. The pectorals have two black cross-bands which extend from the upper part to lower, and the basal part and the outer margin are black.

Fig. 52. Juvenile of *Hirundichthys oxycephalus*. ×1

Fig. 53. Dorsal view of juvenile. ×1

In the young fish the ventral fins are relatively long, having a broad black cross-band, and the basal part and the outer margin black. The dorsal fin has a black patch in the central part. The anal fin is clear. The barbel of the lower jaw is absent. They were attracted to a flood light at night and were swimming close to the surface and skipping or sailing through the air for some distance. Young specimens are economically important off the western coast of Kyushu, being caught in October and November together with the young of *Cypselurus* species.

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Studies on the cellulose-decomposing bacteria found parasitic in the alimentary canal in ruminants and other animals
I. On the isolation of the bacteria

Akira Akashi

Very few studies[8, 13] have been made on the cellulose-decomposing bacteria found parasitic in the alimental canal in ruminants, while a number of studies[1–7, 9–12] made of those in soils. These bacteria, found in the intestine of horses and pigs and in the rabbit's coecum are believed to play an important role in supplying some nutrients to the hosts. Little or none, however, has been known about the strain or strains which predominate in these animals, nor about the effect on the nutrition of their hosts of the product yielded by their activity. In order to throw some light on these problems, a series of study have been undertaken. The present paper deals with a new method of isolation of this type of bacteria.

Results

experiment I

(*1*) *Examination of the routine methods of bacterial isolation.*

As shown in Table 1, liquified cellulose or filter paper is used in all these solid media, and cellulose or filter paper in all liquid media. In these methods, bacterial isolation is effected as in the manner shown in Fig. 1. The cellulose-decomposing bacteria are accumulated on the filter paper in the liquid medium A; bacteria thus accumulated are transferred to the solid medium B and left there till a clear zone is formed; colonies in the zone are transferred back to the liquid medium A, and put back again to a solid medium. The procedure is

Table 1.

Inventers	Ingredients			
Omeliansky	KH_2PO_4	1.0 g	NaCl	trace
	$MgSO_4$	0.5 g	Distilled water	1000 cc
	$(NH_4)_2SO_4$	0.5 g	Cellulose liquid	1000 cc
Dubos	$MgSO_4$	0.5 g	KCl	0.5 g
	$NaNO_3$	1.0 g	$FeSO_4$	trace
Mc. Bee	$(NH_4)_2SO_4$	0.5 g	$CaCO_3$	1.0 g
	KH_2PO_4	0.5 g	Agar	10.0 g
	$MgSO_4$	0.5 g	Cellulose liquid	1000 cc
	NaCl	trace	Cellulose	
Van Viterson	K_2HPO_4	1.0 g	Cellulose liquid	500 cc
	Cellulose	15.0 g	Distilled water	500 cc
	Peptone	5.0 g	$CaCl_2$	0.3 g
	$NaNH_4HPO_44H_2O$	1.0 g		

N. B. Bojanovsky used silicagel for the isolation.

repeated till a pure strain is obtained. It is difficult to isolate the pure strain from among the bacteria which are accumulated by repeated transfer from a liquid medium to a solid medium. Yamada[3)] reported

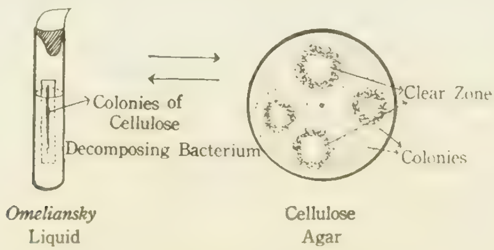

Fig. 1. Scheme showing the routine method of isolating cellulose-decomposing bacteria.

that he failed after two years of trial to obtain a pure culture of cellulose-decomposing bacteria by this method of accumulation. On the other hand, the formation of a clear zone on a solid medium requires a long period of time, depending the thickness of the agar, the pH of the medium and the proportion of the desired bacteria to the contaminating microbes. Moreover, since the clear zone itself is inhibited not only by pure strains but by contaminating strains due to the decomposition of cellulose, a difficulty always results in distinguishing between the genuine and the contaminating bacteria. It is worthy of note that notwithstanding this difficulty, Asai and Ueda[4)] succeeded in isolating several strains of mesophilic cellulose-decomposing bacteria,

and Asai and Yamada[3] isolated thermophilic strains of the same bacteria by a modification of Hungate's method.

(2) *Author's method for isolation of bacteria.*

Examinations of the routine methods disclosed that contaminating bacteria were capable to grow on the saccharide products of the cellulose-decomposing bacteria and that, as cellulose being diffused throughout the agar in the form of gel, many forms of bacteria became naturally mixed up. The fact implies that the liquid cellulose is readily converted into sugar by the bacteria. Hence, a modification was made of adding strips of cellulose to the following medium.

K_2HPO_4 – 1.0 g	$CaCO_3$—2.0 g	$(N_4H)_2SO_4$—10 g	NaCl—trace
$MgSO_4$—0.3 g	Distilled water	—1000 cc	Agar—10.0 g

Both aerobic and anaerobic culture on this medium at 37°C resulted in a better growth of the bacteria as shown in Fig. 2.

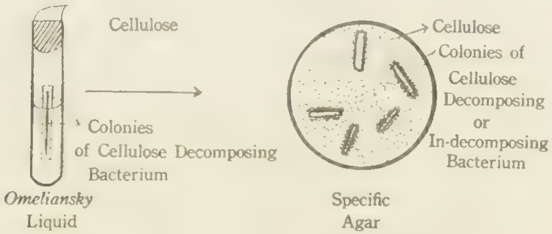

Fig. 2. Scheme showing the author's method in which the cellulose-decomposing bacteria are inoculated on the specific solid medium around each piece of the filter paper.

The isolation procedure finally adopted is as following. The bacteria are accumulated on a strip of filter paper in Omeliansky's medium; in two to three days, there are formed colonies of bacteria around each piece of the paper producing yellow or bluish pigments. A loopful amount of bacteria of each separate colony is tested on the filter paper in Omeliansky's liquid medium for identification of the microbes. It is soon observed that the paper is torn into pieces, suggesting that sample bacteria (both cellulose-decomposing and otherwise) were capable of growing in a medium composed of the aforesaid ingredients without filter paper and that the growth of any contaminating bacteria might be more or less inhibited due to a lack of carbon source supplied from decomposed cellulose. The bacteria thus grown are accordingly inoculated on the aforesaid solid medium with no piece of filter paper in it.

This inoculation was repeated further several times. It will be

observed after two weeks that bacteria in some colonies formed are cellulose-decomposing and those in other colonies are not. Thus, the isolation of the cellulose-decomposing bacteria can be readily carried out within a short period of time by growing them on this filter paper free medium. Ten strains of this form of microbes have so far been isolated from the rumen contents of 15 cows and are under the identification which will be reported later in more detail.

Comment of the experiment I

Most of strains isolated by the author's method are those of mesophilic methane-producing cellulose-decomposing bacteria and several of them are those of thermo-philic hydrogen-producing type. The difference in number between the former and the latter strains obtained may be ascribable to the scarcity of the materials, or may be due to a growth of the former strains to the latter. It is the author's opinion that the filter paper decomposing bacteria isolated by this method may be classified as cellulose-decomposing microbes, although the colonies of these microbes differs from those hitherto reported in that they are bluish or grey without the pigmentation and form tiny circumscribed granules. The strains isolated are mainly the genus *Pseudomonas* which is characterized by its colony being depressed in the agar medium. The colonies formed on the solid medium by bacteria, either cellulose-decomposing or otherwise, are all small in size due to a lack of cellulose as the carbon source. These strains may generally be taken as purified after being grown in diluted form by a plate culture. It is to be mentioned that the microbes thus isolated form colonies of the same type on the author's solid medium, and their cellulose-decomposing power is always been ascertained by a comparable decomposition of the filter paper.

Experiment II

An attempt to reduce the time required for isolation.

As previously reported, a new method described in Experiment I enabled the author to isolate pure strains of the mesophilic and the thermophilic cellulose-decomposing bacteria from the rumen (the former being more abundant than the latter). The time required for the isolation is given in Fig. 3.

Procedure—A. The accumulation of bacteria is followed by the loosening of the filter paper in 2 to 3 weeks (2 weeks in the case of the methane-producing group of the bacteria, 3 weeks in that of the oxygen-producing group).

Procedure—B. The germs thus accumulated are then transferred to a solid medium and left there till a colony is formed; the transplantation is repeated several times till a pure strain is obtained; in most cases it takes 1 to 3 weeks.

Procedure—C. The cellulose-decomposing power of the germs can be checked in 1 to 2 weeks.

In short, for the pure isolation by the present method are required 30 to 50 days in contrast to the other methods by which are required one to several months (in some cases to a year or two). Though it is apparent that the bacterial isolation can be achieved within rather a short time-period, an attempt was made to make every step of culture as short as possible. The period required for (A) and (C) (Fig. 3) is needed for the cultivation in Omeliansky's liquid medium and so can be shortened by the promotion of the bacterial growth, whereas that spent for (B) is indispensable for the aquisition of genuine strains and can not be cut short. Hence, several stimuli suppliments which are known as promoting the cellulose-decomposing activity of the bacteria were added to the medium. The result obtained is given in Table 2.

Table 2.

	Order of decomposing stimulus added power.	Days required for starting decomposition of cellulose	
		Strain I	Strain II
1	Boiled extract of barley seeds	8.	6.
2	Boiled extract of clover stems and leaves	7.	7.
3	Sodium citrate	8.	8.
4	*Azotobacter chroococcum*	11.	11.
5	*Micrococcus*	12.	12.
6	Sodium-sulphate	12.	12.
7	Control	12.	12.

N. B. (1) The germs were determined as growing when the filter began to split.
(2 *Micrococcus*, a genus showing its cellulose-decomposing activity when the Clostridium in the stomach is isolated.
(3) *Azotobacter*—loopful, *Micrococcus*—loopful.

The boiled extracts of barley seeds and of the stems and leaves of the clover, each prepared by boiling a 10 g aliquot in 100 cc of water for 30 minutes is added to the medium in the ratio of 1 : 20 (by volume). The other stimuli supplements are added at a level of the

medium. The decomposition of cellulose is promoted in the medium containing one of the stimuli than in the control. It is clearly shown in Table 2 that the boiled extracts of these clover and barley seed, sodium citrate, and *Azotobactor* are particularly effective as decomposition-promotors. The decomposition-promoting effect of each stimulus varied with its amounts added, as shown in Table 3.

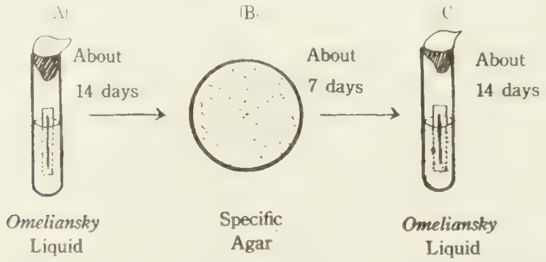

Fig. 3. Diagrammatic representation of the days needed to isolate the cellulose-decomposing bacteria by using the specific agar.

Table 3.

Order of decomposing power	Stimuli added to the medium	Stimulus medium ratio by volume	Number of days for the cellulose to split up no. 3.	no. 4.
1	The boiled extracts of clover leaf stalks	1/20	8	8
		1/10	8	10
2	Sodium-citrate	1/10	8	8
		1/5	14	8
3	*Azotobacter chroococcum*	1 loop	16	15
		5 loops	18	17
4	Control		18	18

Generally speaking, the boiled extracts of clover leaves and that of barley seeds contain a non-nitrogenous substance as their main ingredients, and a lesser amount of amide. The effect of the soluble non-nitrogenous substance may be presumably due to acceleration of citric acid cycle by citric acid formation from non-nitrogenous substances, and that of *Azotobacter* is ascribable to the action of the zooglea. In a word, the decomposition promoting effects of these stimuli may presumably be attributed to the presence of carbohydrate ingredients available as energy sources. This assumption may not be incorrect, because the medium is purposely free from carbon source. Accordingly, several saccharides and nitrogenous matters for efficacy as stimuli suppliments are tested. The results are presented in Table 4.

Table 4.

Order of decompos-ing power	Stimulus supplements added at level of %		Days required for decomposing cellulose	Stage in decompos.
1	Xylose	0.05 %	6	
2	Lactose	0.05 %	7	
3	Dextrine	0.05 %	9	[illegible]
4	Glucose	0.05 %	10	+++
5	*Azotobacter*	1 loop	11	+++
6	Dulcit	0.05 %	12	+++
7	Mannit	0.05 %	13	±
8	Urea	0.05 %	13	±
9	Salicine	0.05 %	13	±
10	Maltose	0.05 %	14	+
11	Saccharose	0.05 %	15	+
12	Sodium nitrite	0.05 %	15	±
13	Potacium nitrite	0.05 %	15	±
14	Control		21	+

++++ Completely decomposed ++ Torn into small strips

+++ Nearly so + So in parts ± Torn into halves.

When xylose, lactose and glucose were added to the medium in concentration of 0.05% and 0.1%, the result as shown in Table 5 was obtained.

Table 5.

Order of decompos-ing power	Stimulus added and the adding ratio	Days required for the cellulose decomposing	Opt. conc.
1	Xylose	5 4	0.05 % 0.1 %
2	Lactose	5 5	0.05 % 0.1 %
3	Detrine	8 7	0.05 % 0.1 %

The effects of sugars are more pronounced than those amides, as shown in Table 6. The promoting effects on cellulose-decomposing power due to the presence of these sugars and of the boiled extracts of barley seeds and of the leaves and stems of the clover are also shown in Table 6 for comparison.

Table 6.

Order of decomposing power	Stimulus-medium	Time required for the cellulose-decomposing (Day)
1	Arabinose (Monosaccharide)	5
2	Xylose (Monosaccharide)	6
3	Saccharose (Oligosaccharide)	6
4	Galactose (Monosaccharide)	6
5	Mannose (Monosaccharide)	6
6	Glucose (Monosaccharide)	7
7	Lactose (Oligosaccharide)	7
8	Boiled extracts of barely seeds	8
9	Dextrine (Polysaccharide)	8
10	Clover-leaf's boiled extracts	9
11	Raffinose (Trisaccharide)	9
12	Mannit (Monosaccharide)	9
13	*Azotobacter*	9
14	Dulcit (Monosaccharide)	9
15	Urea	12
16	Inuline (Polysaccharide)	12
17	Maltose (Oligosaccharide)	12
18	Control	15

Discussion of the experiment II

It is noteworthy that the boiled extracts of barley seeds and clovers and the body contents of the *Azotobacter* was inferior to certain forms of pentose and hexose and that among the saccharides used, pentose is the best promotor of the decomposition of cellulose.

Résumé

In order to obtain a pure culture of the cellulose-decomposing bacteria, an agar medium has been devised which is composed of K_2HPO_4 (10 g), $CaCO_3$ (2.0 g), $MgSO_4$ (0.5 g) $(NH_4)_2SO_4$ (1.0 g), NaCl (trace), distilled water (1000 cc), and agar (1.0 g). Although cellulose is not included in this medium, the cellulose-decomposing bacteria and the other bacteria actually form colonies. In dilute method a pure culture is obtained. When the cellulose-decomposing bacteria which are obtained by this method were inocultated, no decrease is noticed in their activity. The mesophilic cellulose-decomposing bacteria and also the thermophilic cellulose-decomposing bacteria can be cultured on this agar plate, but the latter is rarely seen. A further investigation will

be needed to account for the difference of abundance between these two types.

It has been found in the experiment I that by using the specific agar after culturing in Omeliansky's medium, a pure culture of the cellulose-decomposing bacteria was obtained in a comparatively short time. Shortening of the time of isolation is realized by adopting specific agar, but not by the applying Omeliansky's medium. It takes ordinarilly about 30–50 days to obtain a pure culture, but if time of culture on Omeliansky's medium can be shortened, the isolation will be more readily made. It is to be noted in this respect that carbohydrate is a good stimulus to cellulose decomposition. Especially pentose group and some of hexose group are found excellent.

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Studies on the cellulose-decomposing bacteria found parasitic in the alimentary canal in ruminants and other animals

II. On the isolation of the bacteria by the use of galactose-fuchsin agar and Omeliansky agar

Akira Akashi

Introduction

As described in Report 1,[1] the cellulose-decomposing bacteria in the alimentary canal in ruminants can be isolated in a far shorter period of time when cultured on the author's agar medium (i.e., Omeliansky's medium with agar) compared to time required in any other routine method. The method of isolation which the author used was as follows. A piece of filter paper in Omeliansky's medium was inoculated with an adequate quantity of stomach content, either fresh or air-dried, and kept at 20° to 37°C (30°C suits the purpose in most cases). Two weeks later, when the paper begins to be decomposed and torn into pieces, the liquid part of the medium is diluted with a sterile physiological saline solution 100 to 1000 times its volume; the diluted culture was inoculated on Omeliansky's medium made solid by the addition of agar at a 2 per cent level of agar and cultured at about37°C; the colonies produced are transferred to another piece of filter paper in Omeliansky's medium; the bacteria in the colonies are tested for the cellulose decomposing power. It has been found, as referred to in Report 1,[2] that an addition to the medium of a monosaccharide (xylose, galactose, glucose, etc.) at 0.05 to 0.1 per cent of its volume reduces the time required for the bacterial isolation.

On way of removing the disadvantage (1) of the author's method is to omit the use of Omeliansky's liquid medium for identification. This can be realized when a method is devised which enables us to

distinguish the bacteria under study just as routinely made on Endo's medium. An approach to counteract the disadvantage (2) can be made by furnishing a sufficient amount of cellulose or adequate carbon source to promote the growth of the bacteria if the growth of contaminating bacteria is able to be inhibited by some means.

RESULTS

EXPERIMENT I

Preparation of galactose-fuchsin-added agar medium and discrimination of colonies formed by cellulose-decomposing bacterium on the medium.

The isolation will be simplified if the cellulose-decomposing bacteria can easily be detected by the size and color of their colonies without using Omeliansky's medium; and the bacteria are supplied with sufficient cellulose to promote their growth and make their colonies distinguishable on the one hand, and some substances are added to the medium to inhibit the growth of other forms of bacteria, on the other.

As stated in Report 1,[2] the decomposing power of the bacteria is most promoted by saccharides, monosaccharides in particular, and it is conceivable that other growth of forms of bacteria at the same time can be retarded by the acid product of the increased cellulose-decomposing forms. This method of culture appears to suit the purpose better than those of Omeliansky,[6] Kellermann,[5] and Bojanovsky,[4] in which cellulose agar or silica gel is used and so the growth of contaminating organisms is promoted by a supply of some saccharide from the cellulose gel which is split by the cellulose-decomposing bacteria.

Of the three monosaccharides, arabinose, xylose and galactose, which promote the bacterial decomposition of cellulose, the lastnamed can not be, according to Bergey,[3] decomposed by a majority of enterococci or enterobacteria. It was, accordingly, chosen as substance to be added to the medium, and for the purpose of chromatic discrimination of the bacteria fuchsin was included, as in Endo's fuchsin and lactose-agar. In practice the author's galactose-fuchsin agar medium is prepared as follows:

K_2HPO_4—1.0 g, $(NH_4)_2SO_4$—1.0 g, $MgSO_4$—0.5 g,
$CaCO_3$—2.0 g, NaCL—traceable.

These ingredients are dissolved in 1000 cc of distilled water, added with 0.25—0.5 cc of a galactose stock solution, and further with a sufficient quantity—1.5 cc or so—, of sodium sulfite to deprive the mixture of the red color of fuchsin. The solution thus obtained is

heated to effect better dissolution, filtrated at once at 50°–60°C, and pasteurized for 3 days at 120°–130°C for 20 to 30 minutes a day. This medium should be used within 5 days of preparation, because when left standing at room temperature, it becomes red-colored by oxidation. The way the cellulose-decomposing and the contaminating bacteria form their colonies on the medium will be described below.

(A) The cellulose-decomposing bacteria produce red or pale red pigments when cultured, and form beneath the surface of the medium a round or oval colony which is relatively tiny and granular in shape. The colonies formed on the surface of the medium, are round and moisty. A tiny colony is represented mainly by the *Cellvibrio*, the *Pseudomonas* and the *Cellfalcicull*a, and a larger and granular one by the *Cytophaga*. The *Sporocytophaga* which is incapable of decomposing galactose, forms a white transparent colony on the surface of the agar. It is to be noted, however, that the color of a colony does not depend on the genus of the bacteria but rather on the redox potential at the colony.

(B) Generally speaking, common bacteria do not grow well in this medium except cellulose-decomposing one and their colonies are white-colored in most cases. Eleven intragastrial strains isolated by Akashi show a white color in a period of 24 hours to 5 days and the 4 galactose-decomposing strains among them which usually grow at a rapid rate and turn red on bouillon agar showed a retarded growth on a medium containing nothing but galactose and inorganic salts. But it must be admitted that there are some exceptional cases where the colonies formed on this medium are red-colored. Such cases will be considered on anther occasion. In the majority of cases cellulose-decomposing bacteria are distinguishable from others when cultured on a galactose-fuchsin added agar medium. It is necessary, though, to cultivate these bacteria again on Omeliansky's agar for examination of their cellulose-decomposing power and for preservation of the bacteria thus identified.

Experiment II

The effectiveness of galactose-fuchsin added agar on isolation of cellulose decomposing bacteria as compared with Omelianskys' agar.

Method: Sample A — fresh stomach contents. Sample B — air-dried stomach contents. Sample C — stomach contents inoculated on a filter paper in Omliansky's agar medium — medium O — or on the galactose-fuchsin added agar-medium F — till the paper was decomposed and torn into pieces and then left untreated for a month. These material are each diluted 100-1000 fold and smeared separately or grown

by shake culture on medium F and medium O; the colonies formed are inoculated on filter paper in Omeliansky's liquid medium — medium OmL and in a medium-FeL — composed of: $(NH_4)_2SO_4$ or $NaNO_3$(0.1%) K_2HPO_4 (0.1 %) $MgSO_4 \cdot HO$(0.005 %), $FeCl_3$ (trace) asparagine (traceble) distilled water (100 cc) and cellulose.

The pigments produced on the filter paper were:

(α) group—transparent; cream-white, or white,
(β) group—yolk-yellow, light yellow, fluorescent or orange,
(γ) group—dark, darkish brown, reddish brown.

Table 1. Results of culture.

Number of experiments	Number of isolated strains in three color groups α	β	γ	Ratio in number of isolated cellulose decomposing bacteria to isolated strains put together
(1) A- F-agar shake culture -OmL	0	8	1	9/9 (100 %)
(2) A- O-agar shake culture -OmL	0	0	4	4/9 (44 %)
(3) A- F-agar shake culture -FeL	2	3	1	6/6 (100 %)
(4) A- F-agar shake culture -OmL	0	0	0	0/9 (0 %)
(5) A- F-agar smear culture -FeL	0	0	0	0/7 (0 %)
(6) A- F-agar shake culture -OmL	0	0	0	0/7 (0 %)
(7) A- F-agar smear culture -FeL	0	0	0	0/4 (0 %)

In case the sample A was used, the most easily distinguishable bacteria were those which were cultured on medium OmL followed by the shake-culture on medium F or on medium O alone. The reason why those grown by smear culture were not separable, remains unknown, though it might be ascribable in part to the kind of the material or the condition of the material used.

Table 2. Results of culture.

Number of experiments	Number of isolated strains in three color groups α	β	γ	Ratio in number of isolated cellulose-decomposing bacteria to all isolated strains
(1) B–F-agar-OmL shake culture	0	11	8	19/28 (67 %)
(2) B–F-agar-FeL shake culture	3	7	0	10/28 (33 %)
(3) B–O-agar-OmL shake culture	1	9	8	18/24 (65 %)
(4) B–F-agar-FeL shake culture	1	9	8	18/24 (65 %)
(5) B–F-agar-FeL shake culture	0	4	2	6/24 (25 %)
(6) B–F-agar-OmL shake culture	0	7	2	9/24 (37 %)
(7) B–O-agar-OmL shake culture	0	5	2	7/10 (70 %)
(8) B–O-agar-FeL shake culture	0	1	2	3/10 (30 %)

Table 2 shows that in the case of sample B those which were cultured by shake culture first on medium F and next on medium OmL, or first on medium O and next on medium F and those which formed yellowish colonies (β), were separable in larger numbers than cultured in other ways.

Table 3 shows that in the case of sample C those cultured first on medium C and next on medium OmL and those formed yellowish colonies were unmistakably separable in far larger numbers than those otherwise cultured.

Table 4 was that the galactose-fuchsin added agar medium is more appropriate for the isolation than is Omliansky's agar medium. The superiority of the former over the latter appears accountable from the fact that the cellulose decomposing bacteria are identifiable mainly by the pigments they produced. No change has so far been observed in the pigment produced by the bacteria while isolation and preservation.

Table 3. Results of culture.

Number of experiments	Number of isolated strains in three color groups α	β	γ	Ratio in number of isolated cellulose-decomposing bacteria to isolated strains put together
(1) C–F–agar smear culture–OmL	13	6	1	20/40 (50 %)
(2) C–F–agar smear culture–FeL	10	4	1	15/40 (37 %)
(3) C–O–agar smear culture–OmL	0	0	4	4/9 (44 %)
(4) C–O–agar smear culture–OmL	0	1	1	2/9 (22 %)
(5) C–F–agar shake culture–OmL	5	9	8	22/41 (53 %)
(6) C–F–agar shake culture–OmL	3	7	8	18/30 (43 %)
(7) C–O–agar shake culture–OmL	1	2	23	26/41 (86 %)
(8) C–O–agar shake culture–FeL	1	2	10	13/30 (42 %)

To sum up: Different cellulose-decomposing bacteria producing different pigments can be separated in relatively numerous numbers by culturing in a galactose-fuchsin-added agar medium or in Omeliansky's agar medium.

(2) The white pigmented forms of the bacteria can be separated by a shake culture on Omeliansky's medium followed by culture on the galactose-fuchsin-added agar medium; the yellow pigmented forms by shake culture or smear culture on a galactose-added agar medium in case of a fresh or air-dried sample; and the brown-pigmented forms by shake culture first on Omeliansky's medium and next on Omeliansky's solid medium. (3) The change in color of a colony formed on a galactose-fuchsin-added medium appears to be nonspecific in some cases.

Table 4. Results of culture.

Number of experiments	Number of isolated strains in three color groups α	β	γ	Ratio in number of isolated cellulose-decomposing bacteria to isolated strains put together
(1) C-F-agar smear culture -OmL	13	6	1	20/40 (50 %)
(2) C-F-agar smear culture -OmL	5	9	1	22/40 (55 %)
(3) B-F-agar smear culture -OmL	7	11	1	19/28 (67 %)
(4) A-F-agar smear culture -OmL	0	8	1	9/9 (100 %)
(5) C-O-agar smear culture -OmL	0	2	23	26/30 (16 %)
(6) A-A-agar smear culture -OmL	0	0	4	4/9 (44 %)
(7) C-O-agar smear culture -FeL	1	2	10	13/30 (43 %)

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